MODIFICATION OF PLANT CELL WALLS FOR SECOND GENERATION BIOFUEL PRODUCTION

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For Ryan,

and for Mum, Dad, and Jessica.

Also for Prof. G Paul Bolwell, from his final postgraduate student.

Abstract

The substrate for second generation biofuels is lignocellulosic material obtained from plant cell walls. In the secondary cell wall, lignin and xylan form a waterproof protective network around cellulose, which is made of chains of glucose. Genetic modification of the cell wall has the potential to improve cellulose accessibility and hydrolysis, therefore decreasing the cost and energy input in biofuel production. Working on tobacco, this study aims to improve understanding of cell wall biosynthesis and organisation in order to increase cellulose content and extractability by genetic modification, and additionally by pretreatment with white rot fungus *Phanerochaete chrysosporium*.

Enzymatic saccharification assays showed differences in soluble sugars released from transgenic tobacco lines down-regulated in both lignin and xylan. Significantly, *TOBACCO PEROXIDASE 60* down-regulated line 1074 shows 30% increase in glucose release as compared to the wildtype. Xylan down-regulation by suppression of *UDP-GLUCURONATE DECARBOXYLASE*, which synthesises the xylan precursor xylose, also caused improvement in saccharification. The amount of glucose released from lignin down-regulated lines suppressed in *CINNAMATE-4-HYDROXYLASE* and *CINNAMOYL-COA REDUCTASE* did not increase. The main monosaccharide released from the wildtype lines and all transgenic lines was glucose.

Quantitative real time polymerase chain reaction (qRT-PCR) was used to study the effects of suppressing four cell wall biosynthesis genes on the expression of other genes. The results suggest that the lignin biosynthesis pathway is down-regulated at the transcriptional level in lignin modified lines, while the polysaccharide biosynthesis response differs depending on the position of disruption in lignin biosynthesis. The xylan down-regulated line showed suppression of genes involved in both lignin and cell wall carbohydrate synthesis. However with the exception of the suppression of lignin and xylan synthesis genes in the lignin and xylan down-regulated lines respectively, these results were not reflected in cell wall fractionation analysis. At this level, the lignin supressed lines showed no changes in carbohydrate content while the xylan suppressed line had increased levels of lignin. This demonstrates that cell wall synthesis regulation is not wholly dependent on transcriptional regulation, so there must be multiple levels of cell wall regulation.

White rot fungus *Phanerochaete chrysosporium* naturally hydrolyses and metabolises lignin. It is used to pretreat cell wall material to remove lignin before cellulose hydrolysis, but as yet no research has been published the effects it has genetically

engineered cell wall material. *P. chrysosporium* was incubated with cell wall material from the cell wall modified lines. All lines showed improvement in saccharification after pretreatment. The improvement was as much as threefold in one of the wildtype lines and more than twofold in lines down-regulated in *CINNAMATE-4-HYDROXYLASE* and *CINNAMOYL-COA REDUCTASE*.

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Abbreviations

4CL	4-COUMARATE:COA LIGASE
AIM	Acetone insoluble cell wall material
pg	*Arabidopsis thaliana* line expressing *Aspergillus niger PGii*
pmei	*Arabidopsis thaliana* line over- expressing *PMEI*
COMT	CAFFEIC ACID O-METHYLTRANSFERASE
CCOMT	CAFFEOYL-COA O-METHYLTRANSFERASE
CESA	CELLULOSE SYNTHASE
CSL	CELLULOSE SYNTHASE-LIKE
C4H	CINNAMATE 4-HYDROXYLASE
CCR	CINNAMATE-COA REDUCTASE CINNAMYL ALCOHOL DEHYDROGENASE
EST	Expressed sequence tag
F5H	FERULATE 5-HYDROXYLASE
GC-MS	Gas chromatography-mass spectrometry
G	Guaiacyl-lignin
HCT	HYDROXYL CINNAMOYL TRANSFERASE
H	Hydroxyphenyl-lignin
IRX	IRREGULAR XYLEM
uxs	K326 line with antisense down-regulation of *UXS*
K326	*Nicotiana tabacum* cultivar K326
NVS	*Nicotiana tabacum* v. Samsun
c4h	NVS line with antisense down-regulation of *C4H*
ccr	NVS line with antisense down-regulation of *CCR*
prx	NVS line with antisense down-regulation of *PRX*
C3H	*P*-COUMARATE 3-HYDROXYLASE

PME	PECTIN METHYLESTERASE
PMEI	PECTIN METHYLESTERASE INHIBITOR
PAL	PHENYLALANINE AMMONIA LYASE
PG	POLYGALACTURONASE
PG	POLYGALACTURONASE
QRTPCR	Quantitative reverse transcriptase polymerase chain reaction
RAP2.12	RELATED TO *APATELA* 1.12
RTPCR	Reverse transcriptase polymerase chain reaction
REV	REVOLUTA
SVP	SHORT VEGETATIVE PHASE
SUSY	SUCROSE SYNTHASE
S	Syringyl-lignin
TMEV	Tigr MultiExperiment Viewer
PRX	TOBACCO PEROXIDASE 60
TP60	TOBACCO PEROXIDASE 60
UGD	UDP-GLUCOSE DEHYDROGENASE
UG4E	UDP-GLUCURONATE 4-EPIMERASE
UXS	UDP-GLUCURONATE DECARBOXYLASE
UDP	Uridine diphosphate

1 Introduction

Due to the dwindling fossil fuel resources and global climate change, it is becoming clear that mankind's reliance on fossil energy must draw to an end at some point in the future. Bioethanol is the only alternative energy source which provides liquid fuel, significant as the majority of fossil energy is used as transport fuel. Currently 3.3% of normal unleaded 'petrol' sold in the United Kingdom must be bioethanol (The Renewable Transport Fuels Obligation, 2007). The feedstock for first generation biofuel production must be rich in glucose, fructose or sucrose in order for bioethanol to be energy efficient and economical, but sugary crops are food crops that require good quality land to grow. As a result, land that is needed for food production is being used to grow bioethanol crops (Li et al., 2008). Second generation biofuels do not rely on these high maintenance plants but can be synthesised from any plant or even plant waste (Börjesson, 2009) as cellulose, a polymer of glucose, is present in all plant cell walls. Extracting glucose from cell walls is energy intensive as cellulose microfibrils are part of a strong cell wall matrix consisting of cellulose, xylan and lignin, or pectin in the primary cell wall. Changing cell wall composition to reduce lignin, xylan or pectin content may increase cellulose extractability to make glucose extraction from cell walls less energy intensive. This investigation focuses on four transgenic tobacco lines; all down-regulated in specific genes. These genes encode: *CINNAMATE-4-HYDROXYLASE* and *CINNAMOYL COA REDUCTASE*, key enzymes in the lignin biosynthetic pathway; *TOBACCO PEROXIDASE 60,* a peroxidase known to be involved in lignin biosynthesis; and a *UDP-GLUCURONATE DECARBOXYLASE*, an enzyme which synthesises a precursor to xylan, an important hemicellulose. The aims of this project are to investigate two ways of improving glucose accessibility: reducing xylan and lignin in the cell wall matrix; and use a lignin degrading white rot fungus to pretreat cell wall material. Additionally, the cell wall phenotype and transcriptome of these cell wall modified lines will be analysed in order to further understanding of cell wall biosynthesis and its regulation.

1.1 A history of bioethanol

Bioethanol, the fermentation product of sugars from plant material, is an alternative to fossil energy. Unlike the combustion of fossil fuels, the burning of bioethanol produces energy that is renewable and potentially carbon neutral (Kadam, 2002).

Currently energy from bioethanol is carbon neutral and economically profitable in a limited number of areas (Harari, 2008). Brazil, for example, has established a successful bioethanol industry. In 1985, following the start of the government bioethanol program Proálcool, 95% of new cars in Brazil ran entirely on bioethanol (Rosillo-Calle and Cortez, 1998). Sugar production from sugarcane generates bagasse, an easily burned bi-product in addition to a large store of readily accessible glucose. The molasses are burned to provide energy for the bioethanol refineries, so the whole process is carbon neutral (Rosillo-Calle and Cortez 1998). Despite the success of the Proálcool program in the 1980s, the huge and unexpected demand for ethanol on sugar cane farmers coupled with inflation, the rising price of sugar in international markets and inexpensive gasoline imports led to the slow withdrawal of government support for the bioethanol industry (Rosillo-Calle and Cortez, 1998).

First generation biofuel production relies on sugars produced by plants as an energy store (Figure 1A). Known species of plants that are rich in these sugars are predominantly food crops. Wheat, sugar beet and sugarcane (Li et al., 2008) are widely used but are high maintenance, needing rich farmland which is currently used to meet cities' ever growing demand for food. Converting natural landscapes such as grasslands or rainforest to farmland for bioenergy crops releases a 'carbon debt' of 17 to 240 times more greenhouse gas into the atmosphere than simply continuing with fossil fuels. However, fermenting biomass from waste or non-food crops grown on dis-used farmland would not result in the same 'biofuel carbon debt' (Fargione et al., 2008).

Second generation biofuel can use plant waste or non-food crops as a feedstock. All plants contain glucose in the cellulose microfibrils that, together with hemicelluloses and lignin, make up cell walls. Lignocellulosic material is an ideal source of sugar for bioethanol production (the process is outlined in Figure 1B), as trees and grasses, for example, are rich in lignocellulose and will grow on marginal land that is not suitable for food crops. Many waste products are also rich in lignocelluloses, for example wood chips, sawdust, foodwaste and corn stover. Utilization of local feedstocks and resources is key to maximising the benefits of bioethanol, as this reduces fossil fuel input at the cultivation and transportation stages of the life cycle (Börjesson, 2009).

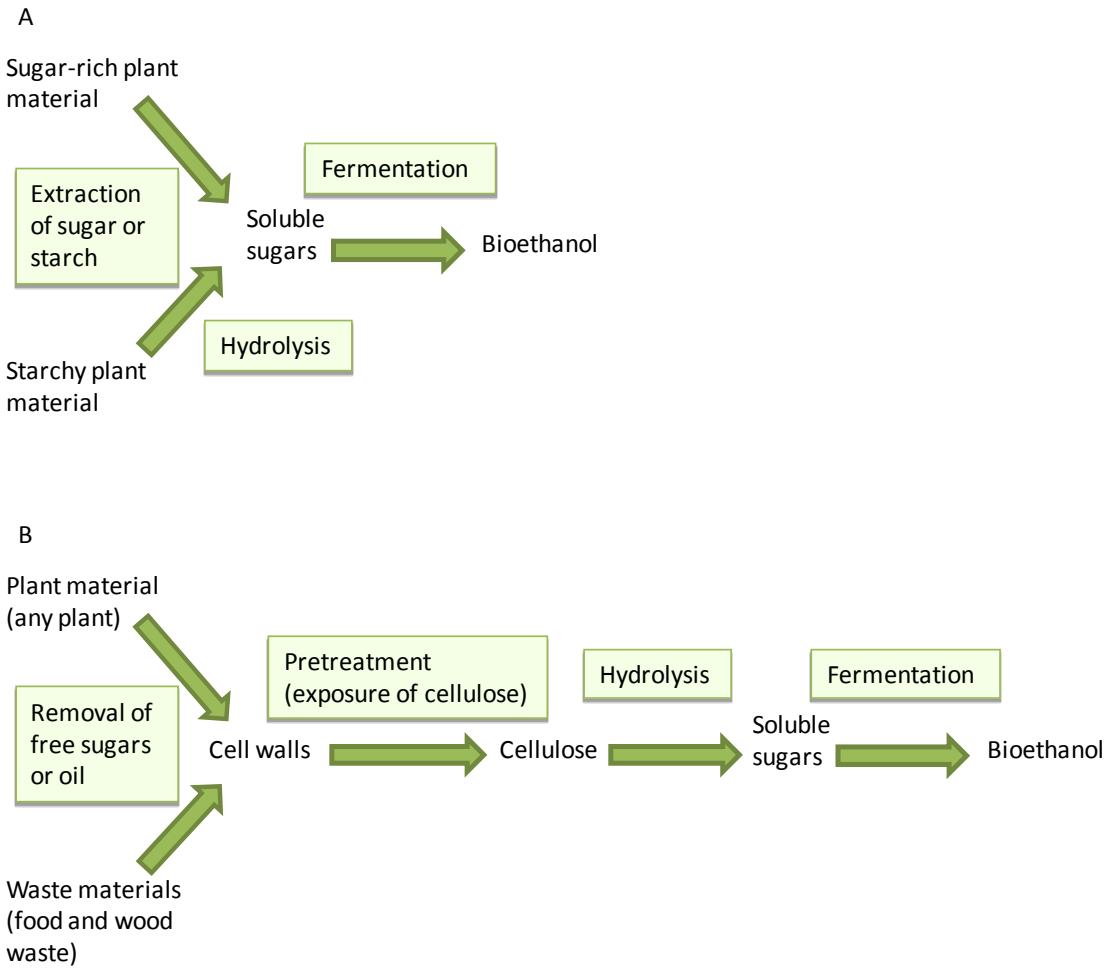


Figure 1 An overview of biofuel production

A The process of generating first generation biofuels. **B** Second generation biofuel production.

However, despite the ubiquity of second generation biofuel feedstock, cell walls have protective and structural functions and are resistant to degradation. The lignin and hemicellulose network surrounding the glucose-rich cellulose microfibrils is insoluble in anything except strong acids or alkalis at high temperature and pressure.

Many industrial pretreatments have been developed to physically remove lignin from the cell walls, exposing cellulose to hydrolytic enzymes to a greater extent. Throughout the work presented here, the plant material was ground using a polytron and by hand under liquid nitrogen. Increasing the area of the substrate available to the enzyme by grinding the tissue is the most basic form of pretreatment (Palmowski and Müller, 2000) and often the starting point for others; although milling to the extent that cellulose is significantly exposed is energy inefficient (Fan et al., 1982). Common pretreatments

are thermal (for example steam pretreatment), in which high temperatures dissolve first hemicelluloses and eventually lignin; acid or alkali, which again work by dissolving hemicelluloses; and irradiation (Grabber et al., 1997; Negro et al., 2003; Taherzadeh and Karimi, 2008; Hendriks and Zeeman, 2009).

Pretreatments, while effective, often have unwanted side-effects. The pretreatments that work by solubilising the cell wall matrix can result in phenolic compounds that have re-condensed. This problem is accentuated in acidic conditions. These phenolics, along with other products such as furfural, inhibit the fermentation process (Keating et al., 2006, Negro et al., 2003, Grabber et al., 1997). The biomass can be washed before fermentation, but soluble sugars can be lost in this way (Mes-Hartree et al., 1988). Pretreatments such as pyrolysis and thermal pretreatments also require energy, commonly sourced from fossil fuel, to work.

Traditionally fermentation is more efficient from hexoses and disaccharides such as sucrose than pentoses. The most commonly used microorganism for the process is the yeast *Saccharomyces cerevisiae,* which naturally metabolises the former molecules (Barnett, 1976). Fermentation of the five-carbon sugar xylose, the second most abundant sugar in cell walls (Hahn-Hägerdal et al., 2007), would automatically increase the sugar yield from any biofuel crop. Bacteria and fungi which metabolise pentoses have adverse side effects; from inhibition by ethanol to the production of many by-products (Chu and Lee, 2007, Hahn-Hägerdal et al*.,* 2007). Stably transformed yeasts that can ferment glucose and xylose simultaneously have been engineered, but to be economically viable they must be able to ferment all other major cell wall sugars, such as arabinose (Hahn-Hägerdal et al*.,* 2006). Currently however fermentation of 6- and 12- carbon sugars continues to be the only process that is suitable for exploitation on an industrial scale (Ha et al., 2011; Kaparaju et al., 2009; Chu and Lee, 2007; Hahn-Hägerdal et al*.,* 2007). In consequence the majority of research focuses on the efficiency of glucose fermentation and accessing, extracting and hydrolyzing cellulose.

1.2 An overview of cell wall structure and synthesis

Cell walls have protective and structural functions making them resistant to degradation. Cellulose microfibrils wrap around the cell to form the framework of cell walls (Figure 2; Somerville, 2006). Hemicellulose crosslinks the cellulose microfibrils via hydrogen bonds, increasing the strength of the cellulose frame (Park et al*.,* 2004). In primary cell walls such as in leaves and fruit, pectin is the third main component of

the cell wall. Non-cellulosic cell wall polysaccharides are synthesised on the Golgi, and then transported to the cell wall by exocytosis, and cellulose is made at the plasma membrane by membrane-bound cellulose synthase proteins (Sandhu et al., 2009). Secondary cell walls, which make up wood and hardy grasses, lack pectin, instead containing the waterproof phenolic macromolecule lignin. These three main constituents form a structured, protective network around the cell (Figure 2 and Figure 3). This network is waterproof, allowing the transport of water through xylem cells.

The cell wall does not merely provide a physical barrier however; it is sensitive to attack from herbivores and microorganisms, as it contains proteins that recognise pathogenic agents and start a defence response (Narváez-Vásquez et al., 2005; reviewed by Collinge, 2009; and Jones and Dangl, 2006).

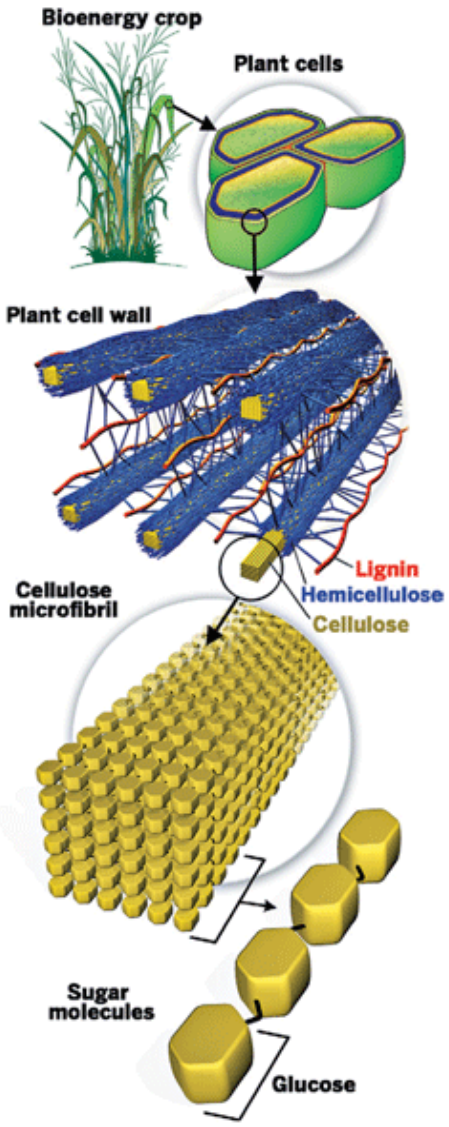


Figure 2 Cell wall structure. Glucose molecules form cellulose microfibrils which are surrounded by a matrix of lignin and hemicelluloses.

Source: Genome Management Information System/ORNL (via Chemical and Engineering News 2008 86 (49):15)

From http://pubs.acs.org/cen/coverstory/86/8649cover2.html, accessed on 16 October 2011

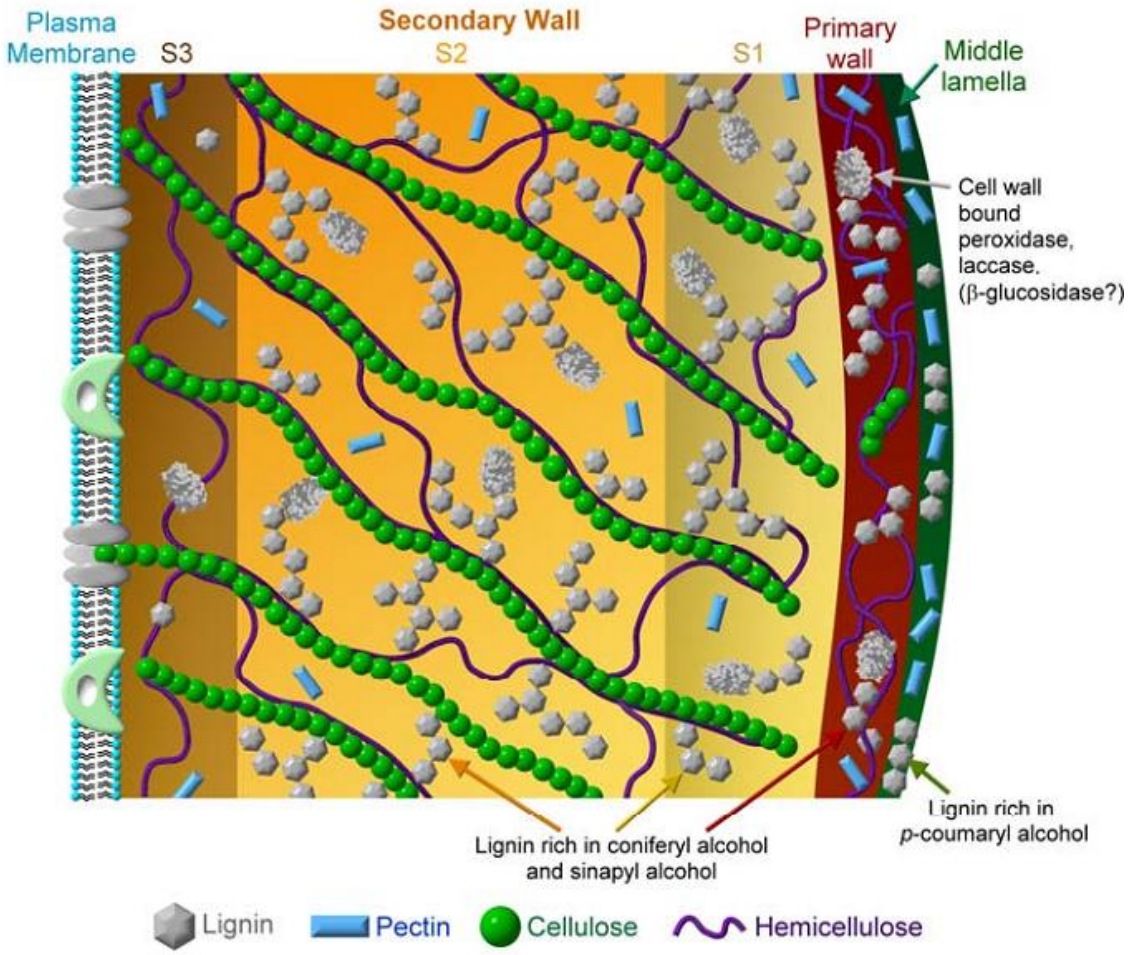


Figure 3 Simplified diagram of the cell wall. Glucose molecules form cellulose microfibrils which are closely associated with hemicelluloses. Lignin cross-links to cellulose and hemicelluloses. Pectin is present at low levels in the secondary cell wall.

Source: Achyuthan et al., 2010.

1.2.1 Cellulose

In plant cell walls, cellulose microfibrils are made up of 36 parallel β-1,4-glucan chains of covalently bonded glucose molecules (chemical structures shown in Figure 4). The microfibrils run in lines which appear to wrap around the cell, providing the framework for the cell wall and giving structural support to the cell itself. Cells in woody tissue contain secondary cell walls which consist of three layers. Cellulose microfibrils in the S1, S2 and S3 layers run in different orientations, ensuring that the cell is strengthened and protected on all sides and from all angles. Cellulose is naturally resistant to hydrolysis because the microfibrils have a tightly packed crystalline structure in which the chains interact via hydrogen bonds and van der Waals interactions. This crystalline structure is insoluble in water, so cellulolytic enzymes have a small surface area to act upon (Buchanan et al., 2000).

Multiple CELLULOSE SYNTHASE (CESA) proteins form structures called rosettes on the Golgi body. They are then transported to the cell membrane. In Arabidopsis, a functional secondary cell wall CESA rosette must contain CESA 4, 7 and 8. Primary cell wall cellulose synthesis requires CESA 1, 3 and 6. As shown in Figure 5, within a rosette there are a number of subunits that catalyse the polymerization of glucose into β-1,4-glucan chains. In Arabidopsis, there are ten subunits. It is therefore assumed that Arabidopsis CESA synthesises ten chains at a time (Somerville, 2006; Mutwil et al*.,* 2008).

As they synthesise β-1,4-glucan chains, the CESA rosettes move along the membrane to create the lines of cellulose microfibrils that form the framework of the cell wall (Figure 5). Their movement is thought to be related to the pattern of cortical microtubules within the cell, as microfibrils in normal plant cells run parallel to the microtubules. However it has been shown that cells with destabilised microtubules have normal cellulose orientation (Himmelspach et al., 2003) and that in normal cells microtubules and microfibrils do not run parallel to each other at all stages of development (Sugimoto et al., 2000). Despite these studies, the idea that the orientation of microtubules and microfibrils are related remains, although the mechanism is unknown (Saxena and Brown, 2008).

UDP-glucose, the precursor to cellulose, is obtained in two ways. Firstly, cell wall associated SUCROSE SYNTHASE (SUSY) localises to cellulose synthesis sites and provides glucose for the CESA complexes by breaking down sucrose into fructose and UDP-glucose (Amor et al*.,* 1995; Salnikov et al*.,* 2001; Persia et al., 2008). Alternatively, starch may be broken down by amylase to provide glucose. Starch grains

disappear rapidly from tissues in which secondary cell wall synthesis is occurring (Bolwell, 1993).

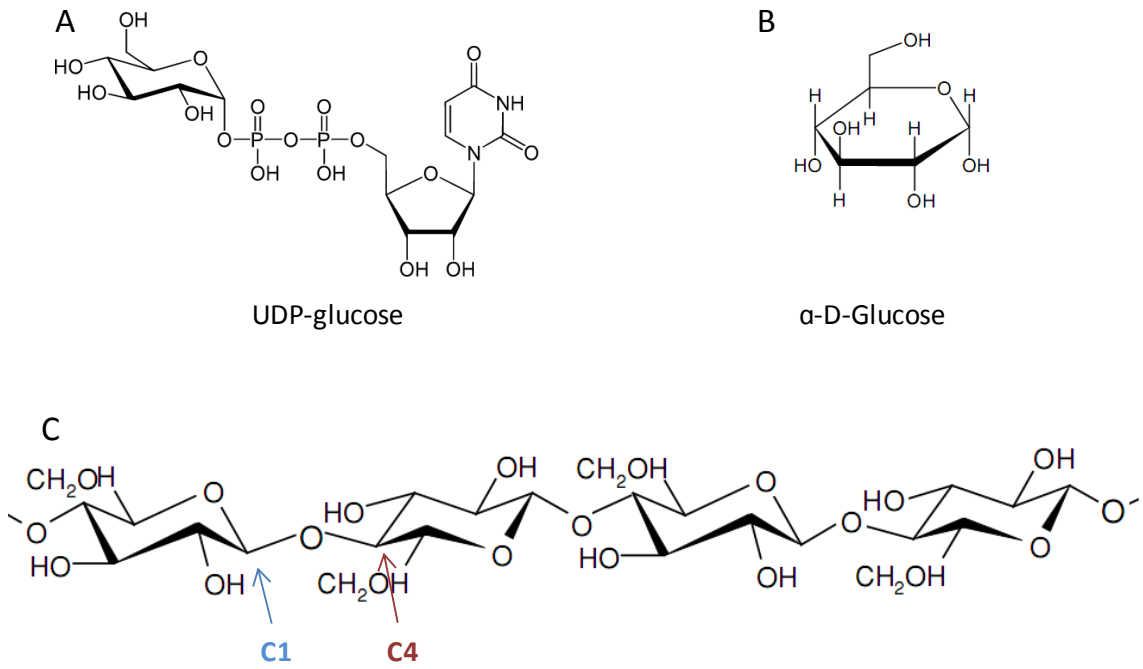


Figure 4 Chemical structure of different forms of glucose

A Uridine diphosphate glucose (UDP-glucose) is the nucleotide sugar form of glucose. All other cell wall monosaccharides are also in their UDP- form prior to assimilation into cell wall carbohydrates. UDP-sugars are transported around the cell by glycosyltransferases.

B α-D-glucose is the sugar that polymerises into chains that make up cellulose microfibrils.

C Glucose molecules linked together by β-(1,4)- linkages which are the linkages found in cellulose. The first and fourth carbons that form the bond are labeled. In cellulose microfibrils, hydrogen bonds form between hydroxyl groups on different chains (adapted from Somerville, 2006).

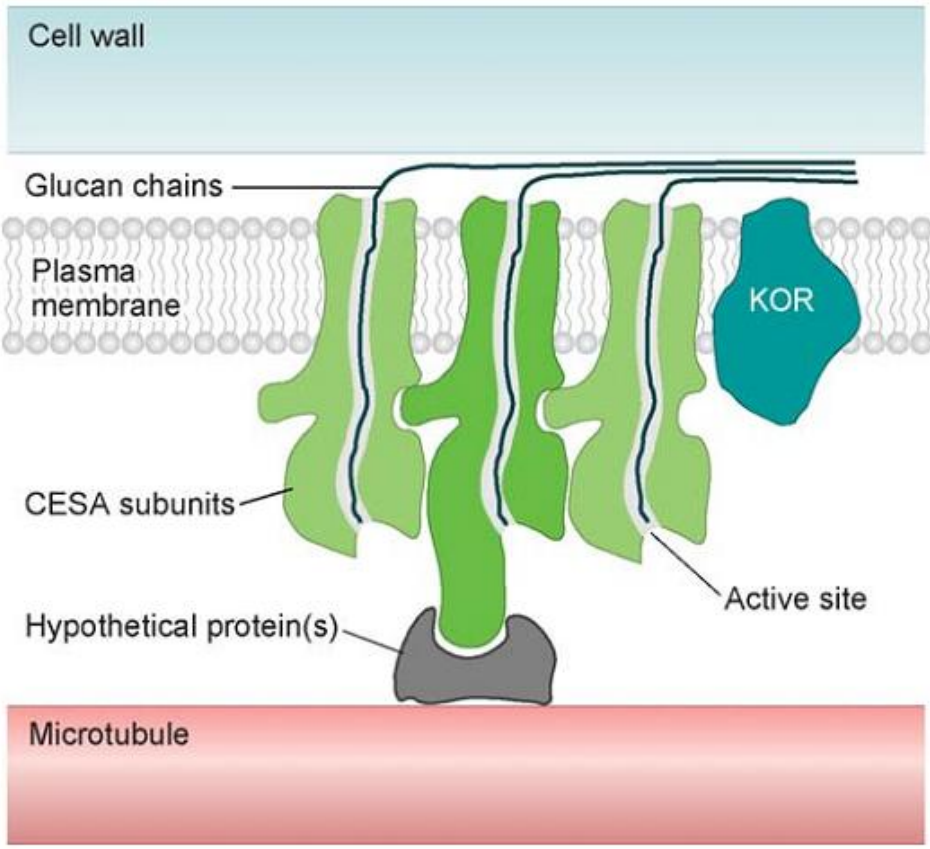


Figure 5 Schematic model of cellulose synthesis. 36 CELLULASE SYNTHASE (CESA) proteins form a rosette on the cell membrane. Cytoplasmic UDP-glucose is incorporated into the glucan chains which feed on to the cell wall. The function of KORRIGAN (KOR) proteins is unknown but *kor* mutants have reduced cellulose accumulation.

Source: Somerville, 2006.

1.2.2 Hemicellulose

Hemicelluloses, also known as cross-linking glycans, link cellulose microfibrils together to strengthen the network (Buchanan et al., 2000) and prevent cellulose hydrolysis. In the primary cell wall, xyloglucan endotransglucosylases rearrange xyloglucans to permit cell growth (Van Sandt et al*.,* 2007). In the secondary cell wall the major hemicellulose is glucuronoxylan, which consists of a β-1,4-linked xylose backbone and sidechains of glucuronic acid residues (Ebringerová and Heinze, 2000). 10% of secondary cell wall hemicelluloses are glucomannan, which contains glucose and mannose (Scheller and Ulvskov, 2010). The chemical structures of hemicellulosic monosaccharides are shown in Figure 6.

The hemicellulose biosynthetic pathway is perhaps the least well characterized pathway of the cell wall polymers. The synthesis of xylose, the direct precursor and 'building block' of xylan is has been established and is illustrated in Figure 8. UDP-glucose is synthesised by SUCROSE SYNTHASE from sucrose or from glucose-1-phosphate by UDP-GLUCOSE PYROPHOSP*HORYLASE.* UDP-GLUCOSE DEHYDROGENASE (UGD) then oxidises UDP-glucose to UDP-glucuronic acid which is either converted to UDP-xylose by UDP-GLUCURONATE DECARBOXYLASE (UXS) or to the homogalacturonan precursor UDP-galacturonate by UDP-GLUCURONATE 4-EPIMERASE (UG4E).

All CELLULOSE-SYNTHASE LIKE (CSL) proteins are able to synthesise (1-4)-β-linked products (Dhugga et al., 2004; Liepman et al*.,* 2005). CSLA synthesises mannan backbones (Liepman et al., 2007) while the CSLC protein family members construct the xyloglucan backbone. The *CSLF6* gene in wheat has also been shown to be a putative β-glucan synthase (Nemeth et al., 2010).

Despite the 35% amino acid sequence identity that CSLD shares with CES proteins (Richmond and Somerville, 2001), its specific function is unknown. Favery et al*.* (2001) showed that the Arabidopsis *CSLD* mutant *kojak* has abnormal root hair growth caused by cells with defective cell walls rupturing soon after root hair growth initiation. They localized CSLD to the endoplasmic reticulum. More recently Bernal et al. (2008) generated *CSLD* mutants which also showed stunted root hair growth and altered wall ultrastructure. Actin and tubulin patterns were disorganized in these mutants. In aspen, *ptrCSLD* shares 45% sequence identity with *ptrCESA*, which includes a distinct cysteine rich region. For these reasons, Bernal et al. (2008), Samuga and Joshi (2004) and Favery et al. (2001) suggest that CSLD has a significant role in the synthesis of an important non-cellulosic cell wall polysaccharide like xylan.

However, no other studies of plants with abnormal xylan have implicated a member of the *CSL* gene family. Several xylan mutants have irregular xylem phenotypes, so the glycosyltransferases whose suppression causes this abnormality have been named *IRX.* Glycosyltransferases are enzymes which transfer a monosaccharide from a nucleotide sugar on to a glycosyl acceptor (Varki et al., 1999).

It is currently thought that there is no single xylan synthase protein, but instead a compex of glycosyltransferases collaborate to synthesise the backbone, side chains and reducing end (York and O'Neil, 2008; Pauly and Keegstra, 2008). IRX8 and IRX9 proteins are involved in the synthesis of the essential sequence of glycosyl residues at the reducing end of all xylan molecules, Xyl*p*-(1,4)-β-D-Xyl*p*-(1,3)-α-L-Rha*p*-(1,2)-α-D-Gal*p*A-(1,4)-D-Xyl*p* (Figure 8; Peňa et al., 2007). IRX7 and PARVUS-3 are also essential for synthesis of this reducing end sequence (Brown et al., 2007). Xylan in mutants of *IRX7,8, 9, 14* and *PARVUS-3* all lack glucuronic acid side branches (Brown et al., 2007). Cumulatively, *IRX10* and *IRX10-LIKE* are essential both for synthesis of the reducing end sequence and addition of glucuronic acid to hemicellulose side-chains in Arabidopsis (Wu et al., 2009). Glucuronyltransferases GUX1 and GUX2 are required for adding glucuronic acid and 4-*O*-methylglucuronic acid branches to xylan in Arabidopsis; their suppression results in xylan with very high extractability due to lack of cross-links with cellulose and lignin (Mortimer et al., 2010).

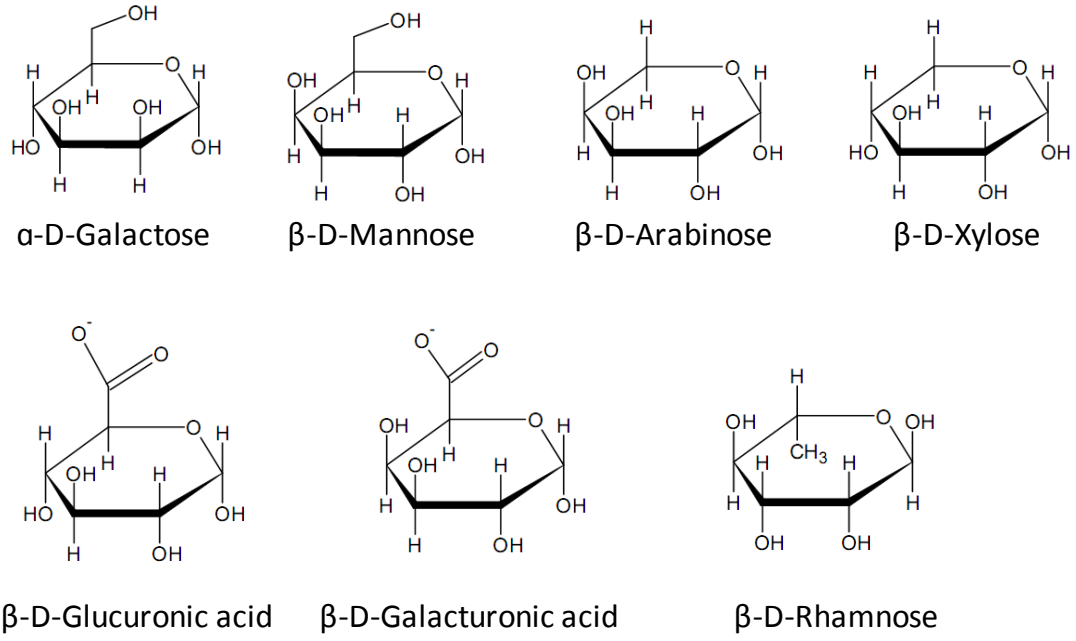


Figure 6 Chemical structures of non-cellulosic sugars.

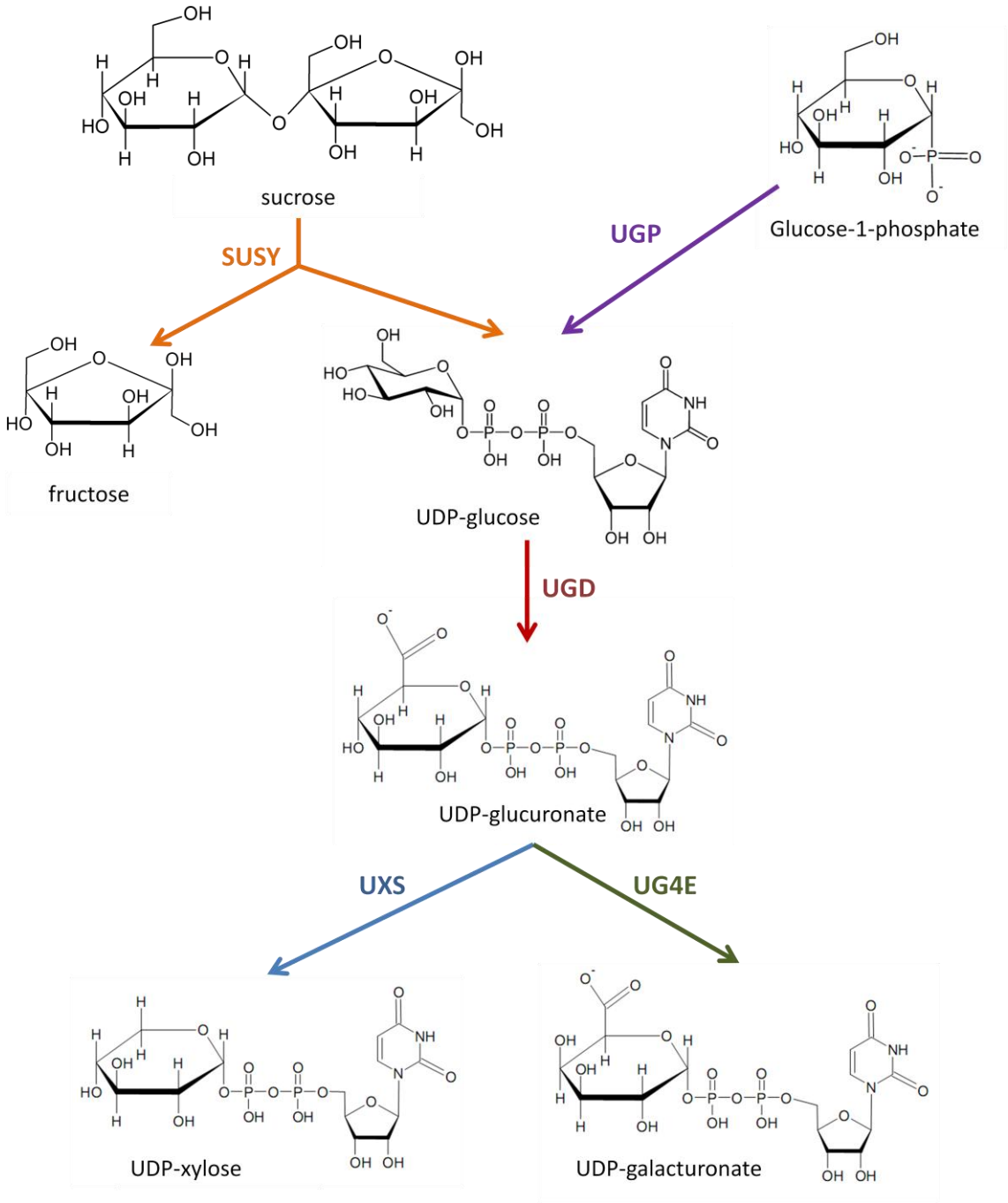


Figure 7 Xylose biosynthesis

UGP, UDP-glucose pyrophosphorylase; SUSY, sucrose synthase; UGD, UDP-glucose dehydrogenase; UXS, UDP-glucuronate decarboxylase; UG4E, UDP-glucuronate 4-epimerase.

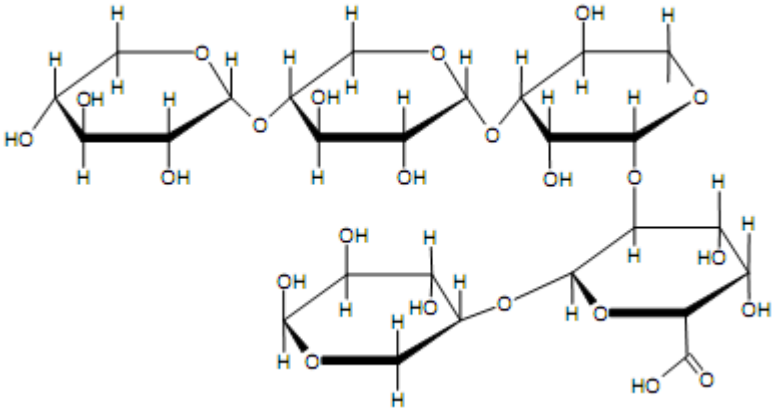


Figure 8 Chemical structure of the sequence found at the reducing end of glucuronoxylan in dicots.

1.2.3 Pectin

Primary cell walls consist of cellulose, hemicellulose and pectin, and though lignin largely replaces it in secondary cell walls, pectin is present in lignified tissue. Pectins are polysaccharides containing 1,4-linked α-D-galacturonic acid (Gal*p*A) residues. Pectin can be divided into three principal groups of molecules: homogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II.

Homogalacturonan (HG) has a backbone of 1,4-linked galacturonic acid (Figure 9) and makes up, on average, 65% of pectins. HG may comprise up to 200 galacturonic acid units, and is partially methylesterified at the C-6 carboxyl group. Xylogalacturonan and rhamnogalacturonan II (RGII) are two structurally modified HGs, which have the same backbone polymer but have side chains containing different sugars. Xylogalacturonan has xylose residues, while RGII has four repeating side chain groups comprising of arabinose, rhamnose, apiose, xylose, fucose, galactose, galacturonic acid and glucuronic acid (Mohnen, 2008; Buchanan et al., 2000).

Rhamnogalacturonan I consists of a backbone with 1,2-linked α-L-Rhamnose alternating with the 1,4-linked gal*p*A residues. Commonly there are acidic oligosaccharide side chains on the rha*p* residues but none on the Gal*p*A residues (O'Neill et al*.,* 1990). The chemical structures of pectic monosaccharides are shown in **Error! Reference source not found.**.

Highly methylesterified homogalacturonan is deposited into the cell wall. It is then de-esterified by pectin methylesterases (PMEs) when the cell has stopped growing. HG

molecules then link together via Ca^{2+} mediated salt bridges to form 'egg box' structures which give the cell wall strength and integrity (Mohnen, 2008). Pectin methylesterase inhibitors (PMEIs) and PMEs act against each other to ensure the correct rate of de-esterification. When the equilibrium is upset by overexpression of PMEIs, the cell wall is more amenable to enzymatic hydrolysis (Lionetti et al., 2010).

It is likely that rhamnogalacturonan II molecules are covalently linked to each other and to HG by borate ester cross-linking (Ridley et al., 2001). Xyloglucan and pectins are also likely to be covalently bonded in the primary cell wall, as they cannot be separated on an anion-exchange column (O'Neill et al*.,* 1990).

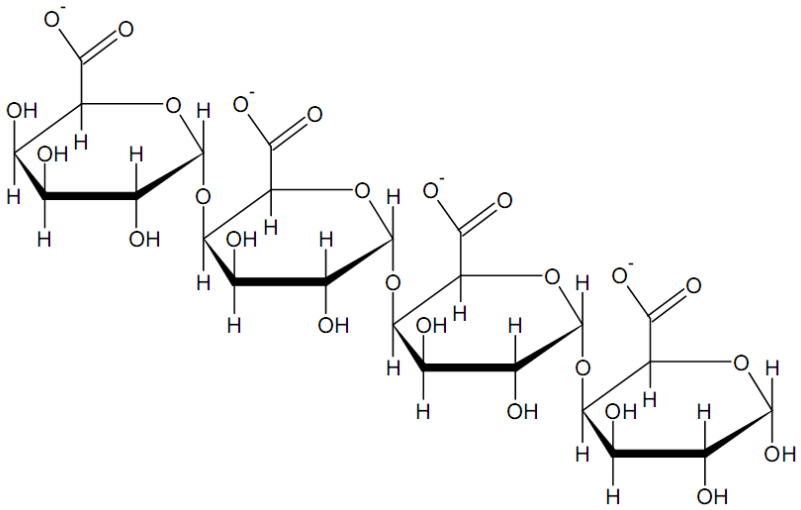


Figure 9 Chemical structure of homogalacturonan. Xylogalacturonan and rhamnogalacturonan II (RGII) have the same backbone polymer of 1,4- linked galacturonic residues but have side chains containing different sugars.

1.2.4 Lignin

In secondary cell walls lignin is the third major component of the cell wall. Lignin is a complex racemic aromatic macromolecule made up of seemingly random arrangements of *p*-coumaryl, syringyl and guaiacyl monolignols. It is hydrophobic, allowing lignin-rich xylem tissue to transport water. This water resistance also prevents hydrolytic enzymes acting on the cell wall components. Lignin is therefore essential for the protection of cellulose microfibrils. It has little energy value and fungi that break it down generally do so in order to access the energy-rich cellulose embedded in it. The phenylpropanoid pathway synthesises all three monomers of lignin (Figure 10) and is conserved between higher plant species (Anterola and Lewis, 2002).

PHENYLALANINE AMMONIA LYASE (PAL) removes the NH_3 group from phenylalanine to form cinnamic acid, and is generally regarded as the first step of the phenylpropanoid pathway (Figure 10; Anterola and Lewis, 2002). However PAL does not appear to catalyse a rate limiting step; Blount et al. (2000) showed that while down-regulation of *PAL* had no effect on CINNAMATE 4-HYDROXYLASE (C4H) activity, an antisense insertion in *C4H* caused PAL activity to reduce. This indicates a feedback loop suggesting that manipulation of *C4H* would be more effective in suppressing lignin synthesis. Consequentially *C4H*, which adds a hydroxyl group to cinnamic acid to form *p*-coumaric acid (Figure 10), is one of the genes targeted in this study. Anterola and Lewis (2002) concluded that C4H regulates carbon flux into the pathway, following detailed analysis of experiments conducted by Sewalt et al. (1997) and Blount et al. (2000) showing that C4H activity and lignin content are linked. Both of these studies involved suppressing *C4H* expression; studies in which *C4H* was up-regulated did not show an increase in lignin despite a greater influx of carbon to the pathway (Anterola and Lewis 2002).

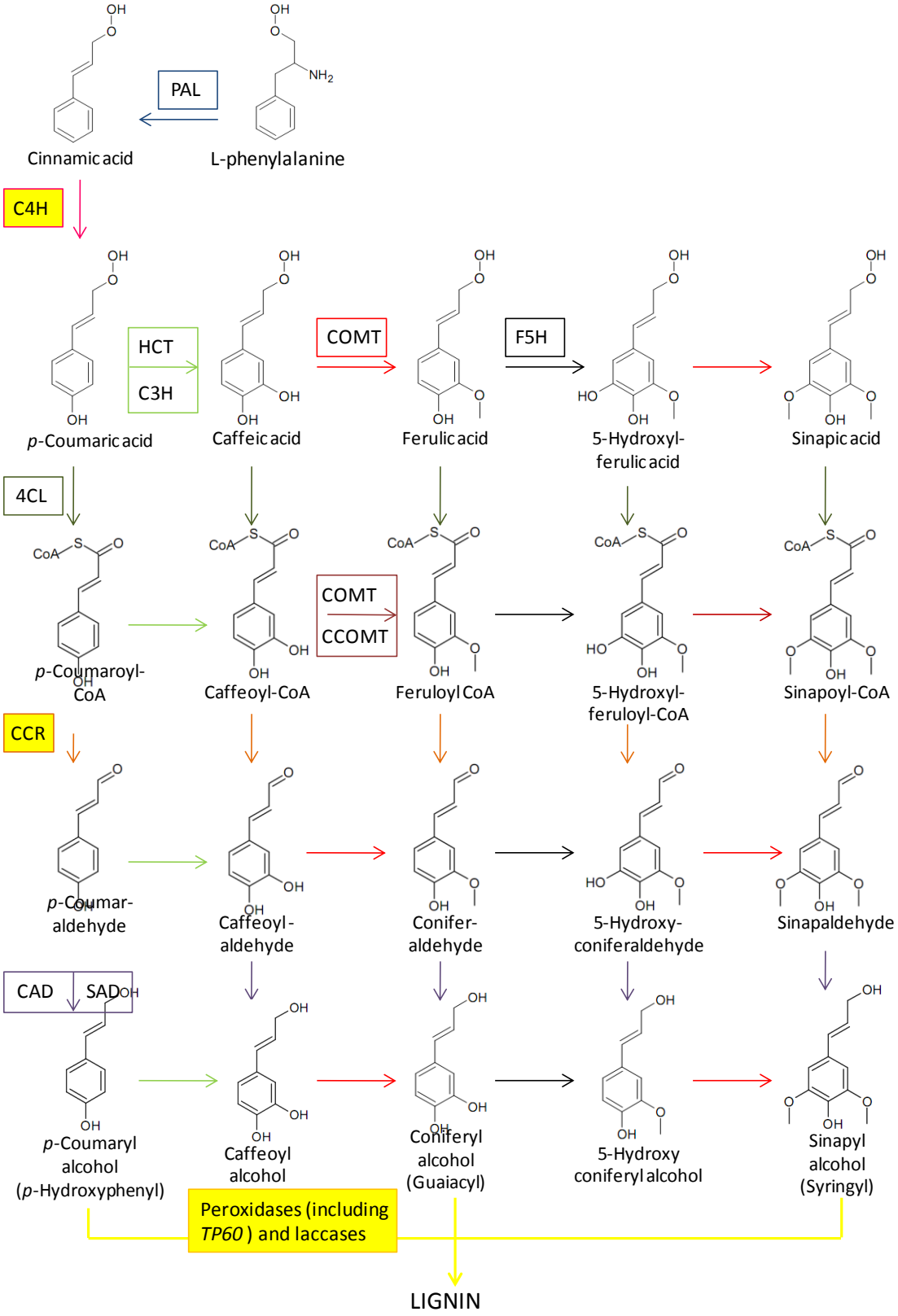


Figure 10 The lignin biosynthesis pathway

Parahydroxylphenyl (P), Guiacyl (G) and Syringyl (S) are the monomers that make up lignin. The arrows are coloured to match the border of the enzyme which catalyses the

reaction they represent. Genes down-regulated in the work presented here are highlighted in yellow.

PHENYLALANIE AMMONIA LYASE, PAL; CINNAMATE-4-HYDROXYLASE, C4H; 4-COUMARATE:CoA LIGASE, 4CL; CINNAMOYL Co-A REDUCTASE, CCR; CINNAMYL ALCOHOL DEHYDROGENASE, CAD; SINAPYL ALCOHOL DEHYDROGENASE, SAD; HYDROXYL CINNAMOYL TRANSFERASE, HCT; P-COUMARATE 3-HYDROXYLASE, C3H; CAFFEIC ACID O-METHYLTRANSFERASE, COMT; CAFFEOYL-CoA 3- O-METHYL TRANSFERASE, CCOMT; FERULATE-5-HYDROXYLASE, F5H.

4-COUMARATE:COA LIGASE (4CL) catalyses the esterification of *p*-coumaric acid into *p*-coumaryl CoA (Figure 10). At this point the phenylpropanoid pathway branches into two separate biosynthesis pathways. *p*-coumaryl CoA is the substrate of two main enzymes: CHALCONE SYNTHASE, which combines *p*-coumaryl CoA with three malonyl CoA molecules to form the basic flavonoid skeleton; and HYDROXYL CINNAMOYL TRANSFERASE (HCT) which synthesises caffeoyl CoA (Figure 10; Besseau et al., 2007; Shadle et al., 2007). HCT and the next enzyme, *P*-COUMARATE 3-HYDROXYLASE (C3H) are required for the allocation of carbon to the parts of the phenylpropanoid pathway that synthesise coniferyl and sinapyl alcohols (Figure 10). CAFFEIC ACID O-METHYLTRANSFERASE (COMT) and CAFFEOYL-COA O-METHYLTRANSFERASE (CCOMT) carry out the final methylation in the synthesis of sinapyl alcohol. The reduction of cinnamoyl-CoA esters to cinnamaldehydes is catalysed by CINNAMATE-COA REDUCTASE (CCR), which is therefore essential for the biosynthesis of lignin and every other downstream product of the phenylpropanoid pathway. CINNAMYL ALCOHOL DEHYDROGENASE (CAD) carries out the reduction that produces monolignols (*p*-coumaryl alcohol, caffeyl alcohol, coniferyl alcohol, 5-hydroxy coniferyl alcohol and sinapyl alcohol) from their cinnamaldehydes. As seen in Figure 10, FERULATE 5-HYDROXYLASE (F5H) adds a hydroxyl group to the fifth carbon of the aromatic ring. It is an essential step in sinapyl alcohol biosynthesis and therefore also required for normal lignin formation. The final products of the phenylpropanoid pathway are monolignols *p*-coumaryl alcohol, coniferyl alchohol and sinapyl alcohol which polymerize into *p*-hydroxyphenyl, guaiacyl and syringyl lignin respectively (Figure 10). In dicots lignin is made up of guaiacyl (G) and syringyl (S) units but monocotyledonous lignin, such as in grasses like alfalfa, also contains *p*-hydroxyphenyl (H).

The next stage of lignin biosynthesis is transport of monolignols to the site of lignification. The process is as yet unknown (Achyuthan et al., 2010). When the

required monolignols are at the site, peroxidases, phenol oxidases and laccases radicalize them by dehydrogenation, releasing an electron from the alcohol group. The electron is delocalised onto the phenolic ring and carbon side chains. The radicalised monolignols then polymerise via combinatorial radical coupling. After each coupling, the molecule must be dehydrogenated again to re-form a radical, which then polymerises and eventually forms the lignin macromolecule. This process is again currently not fully understood. It is possible the polymerization is guided by monolignol radical binding proteins (Davin and Lewis, 2005a,b) which ensure lignin is built, according to a 'template' (Chen and Sarkanen, 2010), in the correct structure and ratio of G, S and H monomers. It has also been suggested that, the monolignols having been transported in the right quantities to the correct site of lignification and radicalised, the polymerisation process is random (Ralph et al., 2009; Vanholme et al., 2010).

The tobacco lines used in this investigation are down-regulated in lignin synthesis genes. *CINNAMATE-4-HYDROXYLASE (C4H*) controls the influx of *p*-coumaric acid into the pathway. This is the precursor to all three lignin monomers (Figure 10). *CINNAMOYL COA REDUCTASE (CCR*) catalyses the reduction mid-pathway of *p*-courmaroyl CoA, feruloyl CoA and sinapoyl CoA to their aldehydes (Figure 10). The peroxidase (*TP60*) is important in the final stage of lignin synthesis, when the monomers are hydrolysed together to form the macromolecule. The monomers are targeted to the final polymerisation stage of lignin synthesis in different ratios depending on species and cell type (Anterola and Lewis, 2002). The mechanism of this and of final polymerisation of lignin is as yet uncharacterised, but involvement of peroxidases has been established (Figure 10; Vanholme et al*.,* 2008). *TOBACCO PEROXIDASE 60*, the peroxidase down-regulated in line *prx* used in this study, is a lignification peroxidase (Blee et al., 2003).

Lignin is linked to cellulose through ether bonds between the hydroxyl group present on glucose which covalently link to one of the carbon atoms in the double bond on the monolignol (Figure 11). These bonds exist between lignin and xylan too, along with ester bridges which form between hydroxyl groups on lignin and the carboxyl group of a glucuronic acid residue in non-cellulosic polysaccharides (Figure 11). Acetal bonds also form between two hydroxyl groups on a hemicellulose or pectin and lignin (Figure 11; Achyuthan et al., 2010).

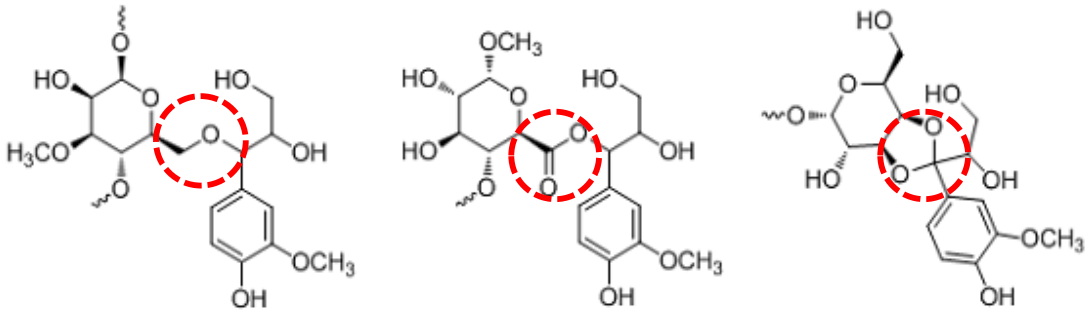


Figure 11 Linkages between lignin and carbohydrates.1 ether bond; 2 ester bond; 3 acetyl bond. Adapted from Achyuthan et al., 2010.

1.2.5 Cell wall synthesis regulation

Several MYB transcription factors have been identified in different plant species as global regulators of lignin biosynthesis (Zhong and Ye, 2009a). ACI and ACII elements (ACCTACC and ACCAACC respectively) have been identified in the promoter sequences of all lignin synthesis genes except *C4H, F5H* and *COMT* (Zhong and Ye, 2009a). Some MYB transcription factors bind to these AC elements, and ectopic expression of Poplar and Eucalyptus MYBs 1, 2 and 4 caused ectopic lignin deposition as lignin synthesis genes were over-induced (Patzlaff et al., 2003; Goicoechea et al., 2005; Bomal et al., 2008).

Additionally, 'masterswitches' including *MYB46, SND1, NST1, NST2, VND6* and *VND7* activate the synthesis of secondary cell wall components such as cellulose and xylan as well as of MYB family members (Zhong and Ye, 2009a).

Modification of cell wall synthesis through manipulation of transcription factors acting as master regulators is a potentially rewarding future branch of biofuel research, though it remains to be demonstrated whether it is possible to intercept lignin biosynthesis elsewhere than the phenylpropanoid pathway (Cook and Devoto, 2011).

1.3 Modification of Cell Walls for Second Generation Biofuel Production

As in any complex composite material, the supramolecular organisation between cellulose, hemicellulose and lignin determines the properties and processing of plant fibres and ultimately the availability of cellulose. Reducing or changing the lignin, pectin or xylan content of the protective matrix by genetically modifying the cell wall biosynthesis pathway could provide a cheaper, easier way of accessing cellulose.

Table 1 shows a summary of published data from research that attempted to improve soluble sugar release from cell walls by changing lignin, hemicellulose or pectin content. The research will be described in more detail in the text below.

Table 1 Comparison of published research into saccharification of plant biomass with modified cell walls

Reference	Species	Modification	Change in saccharification properties as compared to wildtype
Cellulose modification			
Kaida et al., 2009	Poplar (*Populus alba*)	Over expression of cellulase	1.3 fold increase in sugar release from ground xylem tissue *
Lignin modifications			
Chen and Dixon, 2007	Alfalfa (M*edicago sativa*)	*C4H* down-regulation by antisense insertion	No significant difference in saccharification efficiency of stem cell wall material **
		HCT down-regulation by antisense insertion	2.8 fold improvement in saccharification efficiency of stem cell wall material **
		C3H down-regulation by antisense insertion	Twofold improvement in saccharification efficiency of stem cell wall material **
		CCOMT down-regulation by antisense insertion	No significant difference in saccharification efficiency of stem cell wall material **
		F5H down-regulation by antisense insertion	No significant difference in saccharification efficiency of stem cell wall material **

		COMT down-regulation by antisense insertion	No significant difference in saccharification efficiency of stem cell wall material **
Gomez et al., 2010	Tobacco (*Nicotiana tabacum* cv Sansun)	*CCR* down-regulation by antisense insertion	1.5 fold improvement in sugar release from ground stem material*
		CAD and *COMT* down-regulation by antisense insertion	No significant difference from ground stem material
		CAD and *CCR* down-regulation by antisense insertion	1.5 fold improvement in sugar release from ground stem material *
Jackson et al., 2008	Alfalfa (M*edicago sativa*)	*CAD* down-regulation by antisense insertion	Up to 1.7 fold improvement in saccharification efficiency of stem cell wall material **
		CCR down-regulation by antisense insertion	Up to 1.3 fold improvement in saccharification efficiency of stem cell wall material **
Studer et al., 2011	Aspen (*Populus termuloidies*)	Natural variation in lignin content	Sugar release from wood biomass increased significantly with decreasing lignin *
Hemicellulose modifications			
Grabber et al., 1997	Maize (*Zea mays*) cell culture	Treated with hydrogen peroxide to stimulate diferulate formation by 18-40%	12% reduction in sugar release from extracted cell walls *
Lee et al., 2009	Poplar (*Populus alba x tremula*)	GLYCOSYLTRANSFERASE 47 RNAi down-regulation	Up to 1.5 fold improvement in saccharification efficiency **
Mortimer et al., 2010	*Arabidopsis thaliana*	*GLUCURONIC SUBSTITUTION OF XYLAN 1* and *2* T-DNA insertion	2.5 fold increase in % xylan hydrolysis of wood cell wall material **
Kaida et al., 2009	Poplar (*Populus alba*)	Overexpression of xyloglucanase	1.5 fold increase in sugar release from ground xylem tissue *
		Overexpression of xylanase	1.3 fold increase in sugar release from ground xylem tissue *

Pectin modifications			
Lionetti et al., 2010	*Arabidopsis thaliana*	Expression of *POLYGALACTURONASE*	Up to two-fold improvement in saccharification efficiency and 1.5 fold improvement in stem material **
		Expression of *PECTIN METHYLESTERASE INHIBITOR*	Up to 1.6 fold improvement in saccharification efficiency of leaf material and 1.5 fold improvement in stem material **
	Tobacco (*Nicotiana tabacum* petit Havana SR-1)	Expression of *POLYGALACTURONASE*	Up to three-fold improvement in saccharification efficiency of leaf material **
	Wheat	Expression of *PECTIN METHYLESTERASE INHIBITOR*	Up to 1.4 fold improvement in saccharification efficiency of leaf material and 2.5 fold improvement in stem material **

* Results presented as Mol or g of sugar released per g AIM

** Results presented as % of total carbohydrates released by enzymatic saccharification

1.3.1 Increasing cellulose solubility and glucose content

Paracrystalline cellulose has a looser structure with fewer hydrogen bonds than tightly packed, highly structured crystalline cellulose, so there is a greater surface area exposed to potentially cellulolytic enzymes. One way to improve the efficiency of saccharification would be to increase the ratio of paracrystalline cellulose to crystalline cellulose in cell walls (Pauly and Keegstra, 2008). Arabidopsis lines with mutations affecting secondary cell wall components have been screened for saccharification efficiency, and plants with improved sugar release proved to be those with a low relative crystallinity index. The Arabidopsis isoxaben resistant line *ixr1-2* has a mutation in a highly conserved sequence at the C-terminus transmembrane region of CESA3. It has lower crystallinity and saccharification efficiency improved by 51% compared to Col-0 (Harris et al., 2009).

Increasing cellulose deposition in the cell wall would mean a higher yield of sugar from a given amount of biomass, as more sugar would be present. It is possible there is a

compensation mechanism which causes, for example, up-regulated cellulose synthesis when lignin is down-regulated. This is a tentatively drawn conclusion that has not been established, and it is one of the objectives of this project to investigate this fact. Hu et al. (1999) did find that cellulose deposition increases in compensation for reduced lignin due to the suppression of *PAL* and *CCR* in *Populus tremuloides*. By using antisense and sense insertions to downregulate *4CL* and *CONIFERALDEHYDE 5-HYDROXYLASE*, Li et al. (2003) confirmed this idea. The aspen trees they engineered showed 40% less lignin as well as 14% more cellulose as compared to the wildtype. Similarly Chen and Dixon (2007) observed higher total carbohydrate levels in lignin down-regulated lines. In reverse, though still in support of the theory that reducing one cell wall component will cause increased deposition of another, it has been shown that decreasing cellulose synthesis by a mutation in *CESA3* leads to higher lignin synthesis in Arabidopsis (Caño-Delgado et al., 2003).

Hydrolysing cellulose while it is in the cell wall has been shown to the improve saccharification properties of poplar (*Populas alba x tremula*; Kaida et al., 2009). Over-expression of *Arabidopsis thaliana CELLULASE 1* significantly improved sugar release from ground xylem tissue from the transgenic tree. Sugar release was 30% higher in the cellulase over-expresser than in the wildtype.

1.3.2 Lignin modification

Lignin is the main barrier to extracting sugars from cell walls. It is linked through covalent bonds and hydrogen bonds to cellulose, hemicelluloses and pectins, providing a physical barrier between cell wall carbohydrates and the enzymes that degrade them (Figure 3). It is hydrophobic and contains phenolic groups, so it adsorbs these cell wall degrading enzymes, inactivating or inhibiting them (Rahikainen et al., 2011; Berlin et al., 2005; Palonen et al., 2004). Additionally, lignin degradation through physical pretreatments releases phenolic inhibitors that interfere with enzymatic degradation of polysaccharides (Chu and Lee, 2007; Hahn-Hägerdal et al*.,* 2007).

The phenylpropanoid pathway is shown in Figure 10. Many stages in the pathway have been modified, resulting in reduced lignin content (Anterola and Lewis, 2002). Plants in which *PHENYLALANINE AMMONIA LYASE* (*PAL*) has been down-regulated show reduced lignin content, however after analysing trends in *PAL* expression and lignin content, Anterola and Lewis (2002) concluded that *PAL* activity must be halved before any effects are observed. There are other consequences to suppressing *PAL* expression, including stunted growth and deformed, infertile flowers (Bate et al., 1994). Not all of these effects can be ascribed to the decrease in lignin content however, as

the phenylpropanoid pathway synthesises many phenolics (Anterola and Lewis, 2002). The absence or decrease in anthocyanins and other flavonoids seems to contribute to unwanted side effects of lignin down-regulation at this early stage.

CINNAMATE-4-HYDROXYLASE (C4H) is the next enzyme in the phenylpropanoid pathway. Plant lines down-regulated in C4H show no obvious difference in appearance when compared to the wildtype despite having reduced lignin content (Anterola and Lewis, 2002). In 1997 Sewalt et al. repressed *C4H* expression in alfalfa using *C4H* transgene insertions and found that the engineered lines contained less than 20% klason lignin, which is the residue remaining following washes with solvents that remove cellular components and cell wall polysaccharides, compared to the wildtype. They also found that the syringyl:guaiacyl ratio had decreased while plants down regulated for *PAL* had a higher syringyl:guaiacyl ratio (as well as a similar reduction in klason lignin). Down-regulation of *C4H* in alfalfa by antisense insertion did not improve saccharification efficiency of ground stem cell wall material (Chen and Dixon, 2007).

Millar et al. (2007) generated tomato plants down regulated in *C4H* due to homologous sense regulation, where sense transcripts cause RNA interference. Their plants showed many phenotypic effects in addition to the expected reduction in total lignin in the stem. Many of the plants were dwarfed. There were several different leaf phenotypes observed, each with a characteristic fruit mutation. Plants with curly leaves had fewer fruits, which were small and seedless, or produced non-viable seed. Thick waxy leaves were coupled with a reduced number of fruits that bore black marks at their bases. The fruit in many of the mutants contained higher levels of phenolics such as chlorogenic acid and rutin than the wildtype (Millar et al., 2007). Work on *C4H* down-regulation in tobacco by Blee et al. (2001) is described in Section 2.3.3.

HYDROXYL CINNAMOYL TRANSFERASE (*HCT*) was recently identified (Hoffmann et al., 2003) so there are limited published data on its effects on lignin content or saccharification properties. *HCT* silenced tobacco (*Nicotiana benthamiana*) stems contain 25% less klason lignin and an increased proportion of H units than wildtype (Hoffmann et al., 2004). Down-regulation of *HCT* expression by antisense insertion in alfalfa did not significantly improve saccharification efficiency.

Lignin in plants with C3H suppression contains more P (*p*-hydroxyphenyl) units (Ralph et al., 2006). Lignin in *C3H* down-regulated alfalfa plants was up to 65% *P*, while wildtype lignin contains only 1% *P*. Down-regulation of *C3H* in alfalfa also improved saccharification efficiency twofold (Chen and Dixon, 2007).

Plants in which 4-COUMARATE:COA-LIGASE (4CL) has been down-regulated by more than 60% show reduced lignin content than wildtype. Additional penotypes vary between species however, ranging from dwarfism in tobacco (Kajita et al., 1997) to enhanced growth in aspen (Hu et al., 1999). This indicates that 4CL is not a suitable gene to directly manipulate when aiming to reduce lignin in the cell wall matrix (Anterola and Lewis, 2002).

COMT is the only enzyme in the phenylpropanoid pathway that is unique to sinapyl alcohol synthesis. Consequently, plant lines in which *COMT* expression and activity has been suppressed contain fewer S residues in the lignin (Van Doorsselaere et al., 1995; Zhong et al., 1998; Lapierre et al., 1999; Jouanin et al., 2000; Guo et al., 2001; Pichon et al., 2006). There were no effects at all on lignin structure until COMT activity was reduced by 70% or more. At 30% COMT activity, no syringyl monomers were found in lignin in some cases, and syringyl residues were limited in the others (Van Doorsselaere et al., 1995; Atanassova et al., 1995; Jouanin et al., 2000; Guo et al., 2001). Vascular integrity also suffered as the stem strength diminished, indicating that syringyl units are important in maintaining structural support (Anterola and Lewis, 2002). Barnes et al. (1971) and more recently Pichon et al. (2006) found that forage digestibility increased in lines with reduced *COMT* expression, suggesting syringyl monomers are also significant in lignin degradability. Tobacco plants with antisense down-regulation of *CAD* and *COMT* have a lignin content of 74% of the wildtype but show no improvement in sugar release from cell walls by enzymatic saccharification (Gomez et al., 2010).

Two studies in which *CAFFEOYL-COA O-METHYLTRANSFER*ASE (*CCOMT*) was down-regulatied in tobacco give very different results: in one antisense insertion was used to generate *ccomt* lines with <30% residual CCOMT activity and up to 55% lignin content (Zhong et al., 1999); while the other also utilized antisense down-regulation but saw residual CCOMT activity of 85% and a mere 8% reduction in lignin content (Pinçon et al., 2001). The limited effects of *CCOMT* down-regulation on lignification showed by Pinçon et al. (2001) agree with data from other organisms (alfalfa, Guo et al., 2001; and poplar, Meyermans et al., 2000; Zhong et al., 2000). *CCOMT* does not catalyse a rate limiting step (Anterola and Lewis, 2002).

F5H cannot currently be exploited as questions remain as to the specificity of the substrate. Anterola and Lewis (2002) point out that many studies of F5H suggest that there is more than one CYP84 that act on the four potential substrates at this stage in the phenylpropanoid pathway. The inefficacy of *F5H* down-regulation on lignin content

and saccharification properties of cell walls was shown by Chen and Dixon (2007), who synthesised alfalfa lines with reduced *F5H* expression. The *f5h* transgenic line had the same saccharification efficiency as wildtype.

CINNAMOYL CO-A REDUCTASE (*CCR*) suppression results in reduced lignin content in the cell walls which in severely down-regulated lines leads to weakening of the vascular bundle. CCR activity is necessary for normal synthesis of the three monolignols and down-regulating *CCR* causes disruption to lignin composition. Instead of disrupting each monolignol biosynthesis pathway equally, suppressing *CCR* expression causes the syringyl: guaiacyl ratio to increase (O'Connell et al., 2002; Anterola and Lewis, 2002). *CCR* down-regulation in alfalfa caused up to 29% reduction in lignin content and up to 50% improvement in saccharification efficiency (Jackson et al., 2008). Tobacco lines down-regulated for *CCR* also had reduced lignin content at 53% of the wildtype value, and sugar release in this line improved by 50%. This enzyme is clearly a potential candidate for improvement of sugar extractability for bioethanol production.

Plant lines in which *CAD* is suppressed have low levels of lignin. However the effect of down-regulating or mutating *CAD* is not extreme. Two studies on *CAD* down-regulated tobacco lines reduced CAD activity to less than 10% of the wiltype level, but the reduction in lignin content was only 10% (Halpin et al., 1994; Yahiaoui et al., 1998). This is because there is more than one *CAD* present in plants (Anterola and Lewis, 2002) and it is often difficult to assess how complete the suppression of the activity of this protein is achieved. The limited effects of *CAD* down-regulation were confirmed more recently in alfalfa, in which there was a 10% reduction in lignin content and the maximum improvement in enzymatic saccharification was less than 25% (Jackson et al., 2008).

Studer et al. (2011) showed that in poplar, the saccharification efficiency was negatively correlated with lignin content. In other studies the observed trend was not so straightforward; often the most extreme reduction in lignin content did not correspond to the highest saccharification efficiency (Jackson et al., 2008; Kavousi et al., 2010).

1.3.3 Hemicellulose modification

Hemicellulose, the third component of the secondary cell wall, is linked to both lignin and cellulose. Only a few groups have shown that modification of hemicellulose results in increased cellulose hydrolysis. Lee et al. (2009), while investigating the function of a poplar glycosyltransferase GT47, found that reducing glucuronoxylan content in

secondary xylem generated wood that was up to 48% more amenable to cellulose hydrolysis than the wildtype. Over-expression of a hemicellulose degrading xyloglucanase from *Aspergillus aculeatus* in poplar was more effective in improving sugar release from xylem tissue by enzymatic saccharification (50% increase) compared to overexpression of an arabidopsis cellulase or xylanase (both 30%; Kaida et al., 2009).

The final method of modifying xylans in cell walls is reducing the strength of the cross-links between hemicellulose molecules. Two studies have shown that cross-links are vital for cell wall recalcitrance to hydrolysis. In maize, ferulate groups are common side-chains on xylan molecules. Ferulates can dimerise with each other, forming diferulate bonds between xylans. Maize cell cultures were treated with hydrogen peroxide, which stimulated diferulate formation by 18-40%. The increased number of cross links between xylan molecules reduced carbohydrate release from cell walls by 12% (Grabber et al., 1997). Xylan cross-links in arabidopsis were reduced by suppressing the expression of *GLUCURONIC SUBSTITUTION OF XYLAN (GUX) 1* and *2* by T-DNA insertion. GUX is a transferase important in adding glucuronic acid side chains to xylan. Its down-regulation caused almost complete lack of glucuronic acid residues in xylan, and improved enzymatic xylan hydrolysis by 2.5-fold (Mortimer et al., 2010).

1.3.4 Pectin content

The importance of pectin in cell wall cohesion and structure has not been overlooked. Mainly located in the primary cell wall, pectin was found to be significant barrier to cellulolytic enzymes access to cellulose. Pectin is hydrolysed by plant pathogenic fungi by polygalacturonase (PG) enzymes in order to access the cells (Buchanan et al., 2000). When expressed *in planta*, *Aspergillus niger PGII* causes a 25% reduction in uronic acid content, reduces the lebels of deesterified homogalacturonan (Capodicasa et al., 2004) and improves saccharification efficiency twofold (Lionetti et al., 2010).

Homogalacturonan is released from the Golgi body in a highly methylesterified form. Pectin methylesterases (PME) demethylesterify homogalacturonan into a form that is incorporated into the cell wall and becomes integral to its structure. Demethylesterified homogalacturonan can form Ca^{2+} bonds with other pectin molecules or may be degraded by enzymes such as plant PGs in order to reduce the rigidity of the cell wall, for example during fruit ripening (Mohnen, 2008; Pelloux et al., 2007). PME inhibitors (PMEIs) prevent PME activity, therefore limiting cross-linking in the primary cell wall. *Arabidopsis thaliana* lines which overexpress the endogenous *PMEI1* or *PMEI2* have 16% more methylesterified homogalacturonan than wildtype (Lionetti et al., 2007).

PMEI overexpressers also have a saccharification efficiency of up to 50% more than wildtype (Lionetti et al., 2010). The same transgenesis also improved saccharification efficiency of tobacco leaves and wheat stem material.

1.4 Xylem structure and xylogenic tissue

Tobacco plants are used in this study because they are considered to be a model woody organism (Chabannes et al., 2001). Tobacco is easy to transform and has a short life cycle relative to other 'woody' plants, for example poplar. 'Woody' material in plants is the secondary xylem, which is synthesised after primary growth has ceased. During primary growth, the plant grows taller and the stem lengthens. At this stage, the xylem is primary xylem. Secondary growth occurs when the stem becomes thicker; the secondary xylem forms the 'woody' tissue which gives stems strength (Etchells and Turner, 2009). This secondary xylem was the tissue used for the research presented here.

Vascular tissue is made up of phloem, which transports organic compounds, and xylem, which transports water and mineral nutrients. It forms a continuous system from root tips to the leaves, where it divides many times to ensure every part of the leaf has a supply of water and sugars (Myburn and Sederoff, 2001). There are two water conducting cell systems in xylem tissue. Both tracheids and vessel elements have cell walls with high lignin contents, so the water remains within the cells and the plant is given structural support. When the cells are mature, they form hollow systems of interconnected, non-living cells through which water can flow without crossing the hydrophobic secondary cell wall (Figure 12; Hopkins, 1995). Vessel elements are more abundant that tracheids, comprising 70% of xylem tissue in tobacco (Hepworth and Vincent, 1998).

Tracheid cells are long and thin, in tobacco they measure between 2-4 mm long (Hepworth and Vincent, 1998). As seen in Figure 12, they are connected to one another by pits to form tubes that run longitudinally up and down the plant. Pits are areas between two cells where the secondary cell walls are absent and only the primary cell wall and middle lamella form the pit membrane that divides the cells. Water can cross freely over the pit membrane. Some pits have 'borders', where the secondary cell wall is slightly extended and the pit is bridged by a swollen region of the pit membrane called a torus. The torus moves according to water pressure to block entry of water into certain cells if the water pressure is too high (Hopkins, 1995).

Vessel elements are longer than tracheid cells, but like tracheids are arranged end to end longitudinally within the xylem. When the vessels are mature the ends of each cell form openings called perforation plates (Figure 12) to allow free movement of water within the column. The wall surrounding the perforation plate is very thick, containing lignin with very little cellulose (Milburn, 1979).

Solutes are not only transported in the water conducting cells but are stored in the xylem in parenchyma cells. These cells also have thick secondary cell walls. Structurally important are the sclereids and fibre cells, which wrap around the cortex to form the 'woody' tissue (Lev-Yadun, 1997). Fibres are elongated, non-living cells (Hopkins, 1995) which are heavily lignified and provide structural strength to the plant. Sclereids are so strong as to protect from herbivores; it is the lignified sclereids that make nutshells so strong (Lev-Yadun, 1997).

Irregular xylem phenotypes have collapsed vessel elements and are usually caused by cell wall modifications. Down-regulation of structurally important components causes cells to buckle under the pressure of water transport. Transgenic Arabidopsis lines deficient in cellulose and xylan synthesis have irregular xylem (*irx*) phenotypes (Turner and Somerville, 1997; Taylor et al., 1999; Taylor et al., 2003; Peňa et al., 2007). Cell wall modification can cause increased vessel element size; xyloglucan rearrangements cause larger vessel elements in aspen (Nishikubo et al., 2011).

The irregular xylem phenotype can cause dwarfism (Taylor et al., 2003; Brown et al., 2005; Brown et al., 2007, Peňa et al., 2007), changes to the vascular structure do not need to result in major physical abnormality. Arabidopsis *irx* mutants with reduced cellulose content had phenotypes ranging from slightly shorter stems to no differences from the wildtype (Turner and Somerville, 1997). Nishikubo et al. (2011) described aspen trees which had large vessel elements due to reduced hemicellulose content, and were slightly shorter than the wildtype but otherwise healthy.

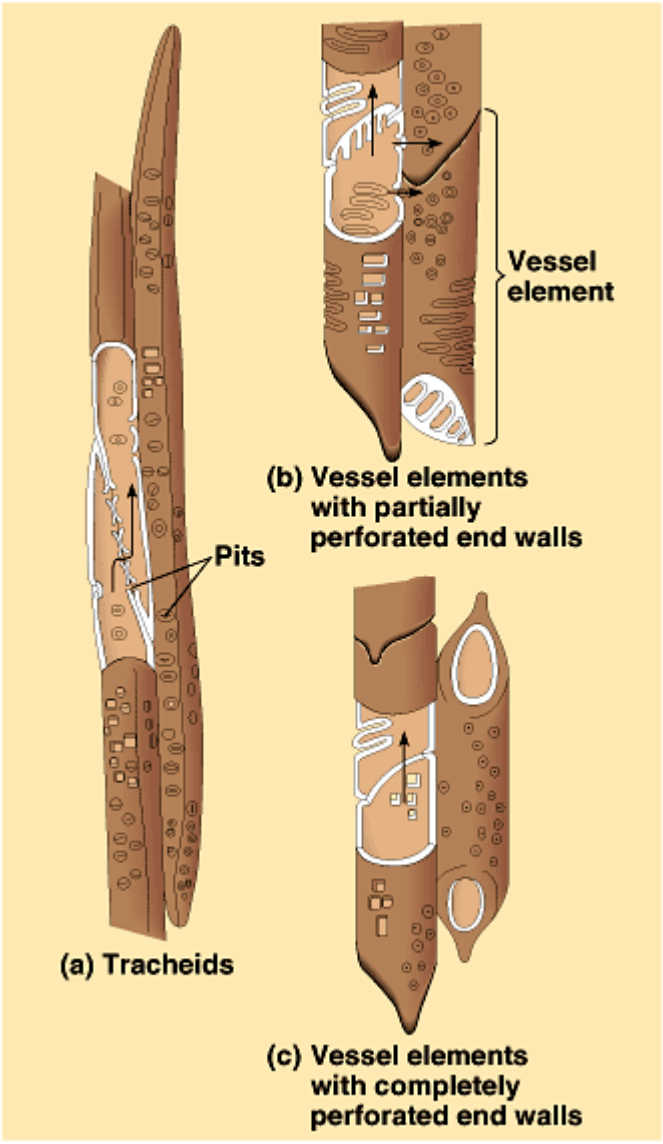


Figure 12 Cells in xylem tissue. Tracheids and vessel elements both transport water. The cells form a continuous column, due to pits in tracheid membranes and perforated vessel element walls.

Source: Pearson Education Inc, via http://www.bio.miami.edu/dana/226/226F09_5.html accessed on 17 October 2011

1.5 Cell wall degrading fungi

1.5.1 Introduction

Cellulose and lignin are the two of the most abundant plant polymers on earth. Despite this, animals largely avoid eating the secondary cell wall in which these polymers exist, preferring the soft pectin and cellulose in the primary cell walls of fruits and leaves. This is because, as outlined above in Section 1.2, lignin in the secondary cell wall prevents, by its physical presence and the water-proof structure (Figure 3), digestion of itself and the cellulose it is linked to by water-soluble enzymes (Hu et al., 1999).

Lignocelluloses can be pretreated in various ways in order to ease cellulose accessibility. However, chemical and physical pretreatments are expensive and environmentally damaging and often inhibit the fermentation process (Grabber et al., 1997; Keating et al., 2006; Negro et al., 2003). The use of microorganisms that naturally degrade or metabolise lignin, leaving cellulose exposed and intact, represents a valid alternative to aggressive and expensive pretreatments.

1.5.2 Cell wall degrading organisms and their modes of action

Microorganisms that can depolymerise lignin are of extreme interest to the biofuel industry because lignin is the main barrier to cellulose hydrolysis. If a fungus or bacterium could strip lignocellulose down to cellulose with minimal energy input, there would be no need for expensive and environmentally unfriendly chemical and physical pretreatments.

There are two groups of bacteria which can degrade cell walls: tunnelling bacteria and erosion bacteria. They both produce cell wall degrading enzymes and digest a path through wood, resulting either in tunnels or erosion troughs in the wood surface (Clausen, 1996; Björdal and Nilsson, 2008).

Soft rot fungi, for example *Tricoderma reesei*, digest a path through wood in a similar way to tunnelling bacteria. From the cell lumen the hyphae pierce the cell wall and run alongside cellulose microfibrils, releasing enzymes in order to de-polymerise space to grow into, as shown in Figure 13. Their preferred substrate are polysaccharides rather than lignin (Hammel et al., 2002).

Brown rots metabolise cellulose, so that lignin is left after the fungus colonises the wood material. The mechanism of degradation is via a decay agent that penetrates

further than the fungal hyphae themselves (Figure 13; Dashtban et al., 2009). Decay agents are small non-enzymic molecules which are discussed in 1.5.3.

White rot fungi are the only organisms that not only de-polymerise lignin in order to access cellulose, but can metabolise lignin as a primary carbon source if necessary (Dashtban et al*.,* 2009). In these instances the lignin is stripped from the cell wall material, leaving the white cellulose behind as shown in Figure 14. Lignocellulose has such a large, complex structure that it cannot be absorbed into the cell to be digested (Kirk and Ferrell, 1987). The fungi and bacteria that de-polymerise and digest it secrete extracellular enzymes such as lignin peroxidase. All the microorganisms mentioned above de-polymerise lignin in the same way as white rot fungi. As lignin is an inaccessible hydrophobic molecule, enzymes are incapable of acting directly on it. Lignin peroxidases generate free radical species which attack the aromatic rings in the monolignols. The process is described below.

1.5.3 Fungal cell wall degrading mechanisms

Phanerochaete chrysosporium strain 24725 (a kind gift from Laurence Davin; Washington State University) was characterised by Alan Buddie (CABI, Egham, UK) and used in this study. *P. chrysosporium* is a white rot fungus from the order *Polyporales* in the class *Basidiomycetes*. Its reproduction is usually asexual, although homothallic systems can exist (Alic et al., 1987). This strain degrades lignin (Camarero et al., 1994) and produces cellobiose dehydrogenase (CDH; Wood and Wood, 1992).

P. chrysosporium can metabolise monosaccharides and polysaccharides as well as lignin. When grown with cellulose as the sole carbon source, a fully competent ligninolytic system is expressed (Sharay et al., 2008). It has proven to improve the glucose extractibilty from lignocellulosic biomass (Bak et al., 2009; Shi et al., 2009; Salvachúa et al., 2011; Zeng et al., 2011; Shrestha et al., 2008) and a summary of the results of previous pretreatment investigations using *P. chrysosporium* is presented in Table 2. White rot fungi such as *P. chrysosporium* use a wide range of glycoside hydrolases that act on cellulose and xylan: endoglucanases that break glycosidic bonds in the middle of a molecule, cellobiohydrolases that act on chain ends and β-glucosidases that clean up stray cello-oligosaccharides that are left over (Dashtban et al., 2009). All the glycoside hydrolases work with cellulose binding molecules when hydrolysing crystalline cellulose. The binding molecules specifically bind to cellulose by an unknown mechanism and facilitate hydrolysis of the glycosidic bonds that link the glucose monomers together (Dashtban et al*.,* 2009; Wilson, 2009).

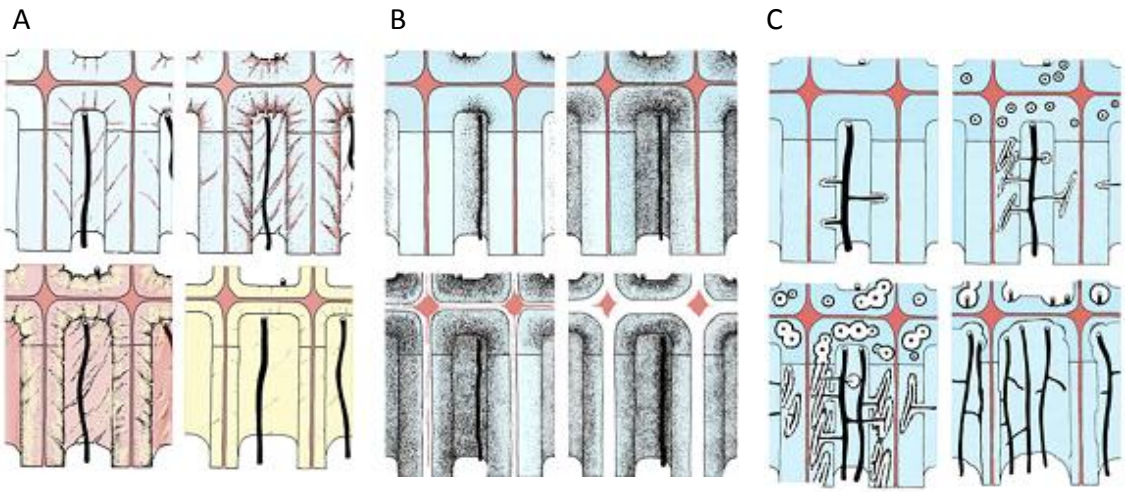


Figure 13 Schematic drawings showing micro-morphological features of different decay types. The stages of decay by three fungi are shown clockwise from top left in each figure (adapted from Schwarze, 2007).

A Decay by brown rot *Fomitopsis pinicola*. First, low molecular weight substances are secreted by hyphae and diffuse into the cell wall. Next, enzymes have penetrated into the entire secondary wall, breaking down hemicelluloses and cellulose. The decay leads to the formation of numerous cracks and clefts within the secondary wall. In the advanced stages the S_3 remains intact, including a matrix of modified lignin.

B Selective delignification by white rot fungus *Heterobasidion annosum*. First, low molecular weight substances (shown as dots) diffuse into the secondary wall from hyphae growing in the lumen. These initiate the degradation of hemicellulose and lignin within the secondary wall. Lignin and pectin are preferentially degraded so in the advanced stages the cells become separated from one another.

C Decay by soft rot *Kretzschmaria deusta*. First, hyphae grow into the secondary wall. In the wall, the hyphae grow parallel to the orientation of the cellulose microfibrils in the S_2 layer. Degradation of the cell wall around the hyphae leads to the formation of cavities with conically shaped ends. In the advanced stages of decay the secondary wall is nearly completely broken down but the guaiacyl-rich compound middle lamella persists.

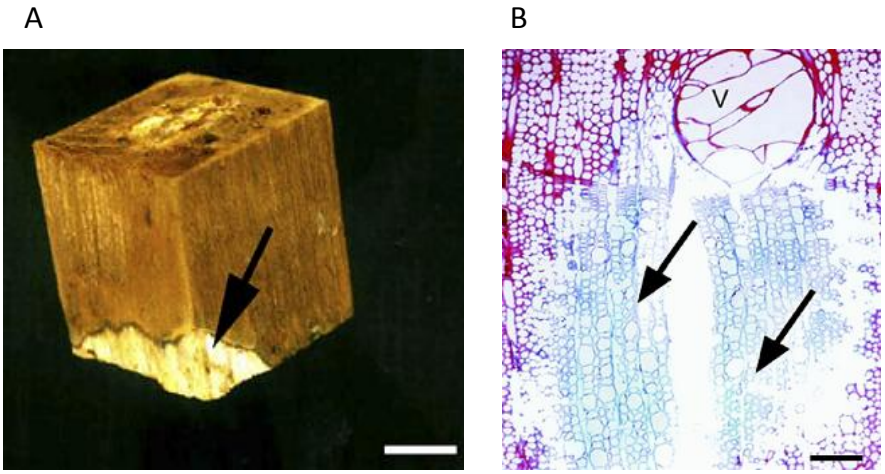


Figure 14 Examples of white rot selective delignification (adapted from Schwarze, 2007).

A Wood block of robinia artificially incubated with white rot fungus *Perenniporia fraxinea*, the bottom of the wood block was exposed to the fungus. The white area pointed out by the arrow is cellulose and hemicelluloses that have been stripped of lignin. Bar: 2 cm.

B Transferse section of oak wood infected with *Grifola frondosa*, stained with safranine and astra-blue. Lignin is stained red. The arrows point out delignified, light-blue cell regions which have been delignified by the fungus. Bar: 50 µm.

Microorganisms use the hydrolysis of cellulose and xylan to generate reactive oxygen species that can be used in turn to de-polymerise lignin. Cellobiose dehydrogenase (CDH) oxidises cellodextrans and other products of incomplete cell wall degradation using a wide range of electron acceptors including Fe^{3+} and oxygen, producing H_2O_2 and supporting the hypothesis that Fenton chemistry is instrumental in cell wall degradation (Hammel et al., 2002). CDH is made up of an FAD-containing domain which bonds strongly to the substrate and contains the catalytic site, and a haem group which has an undefined function but it essential for efficient function of the enzyme (Henriksson et al., 2000). The by-products of this reaction are benzoquinone or dichlorophenol indophenols. The Fenton reaction is as follows:

$$H_2O_2 + Fe^{2+} + H^+ \rightarrow H_2O + Fe^{3+} + \bullet OH.$$

Additionally, *P. chrysosporium* synthesises veratryl alcohol (VA) from phenylalanine during secondary metabolism. Lignin peroxidase synthesis is induced by VA, which also stabilises it against H_2O_2 and free radical species. VA is then oxidised by lignin peroxidase, which is a cytochrome P-450 that transfers electrons from veratryl alcohol onto another peroxide, generating veratryl alcohol free radicals (Haemmerli et al.,

1987). These radicals and others, including •OH which are generated by the Fenton reaction, attack the aromatic rings of the lignin monomers (Hammel et al*.,* 2002). The radical adds itself to the ring, starting a number of possible strings of reaction which may result in a lignin phenoxy radical being produced, or release of a hydroperoxyl radical. Both of these can further depolymerise the rest of the lignin molecule.

White rot fungi have proved efficacious in pretreatment of lignocellulosic biomass prior to saccharification. They are effective on a range of starting material including wheat straw (Hatakka, 1983), bamboo (Zhang, 2007) and wood chips (Hwang, 2008).

Table 2 Summary of published research on pretreatment of plant biomass with *Phanerochaete chrysosporium*

Reference	Material	Pretreatment time	Results for:	Control	Pretreated
Bak et al., 2007	Ground rice straw	15 days	Cellulose (%)	35.7	30
			Lignin (%)	19	15
			Saccharification efficiency (%)	20	55
Shi et al., 2009	Cotton stalk	14 days	Cellulose (%)	37	39
			Lignin (%)	29	21
Salvachúa et al., 2011	Wheat straw	7 days	Cellulose (%)	29	20
			Lignin (%)	24	24
Zeng et al., 2010	Wheat straw slurry	7 days	Cellulose (%)	37.7	24.2
			Lignin (%)	19	12.3
			Saccharification efficiency	3.5 fold increase after pretreatment	
Shrestha et al. 2008	Corn fiber	6 days	Lignin	41% reduction	
			Ethanol production (g/100g corn)	1.7	3

1.6 Transcription profiling as a guide to discover novel genes and to study unintended effects of genetic engineering

Transcriptomics, the analysis of gene expression at a genome-wide level, can be used to uncover genes involved in cell wall synthesis and their regulators. Potentially, whole pathways can be modelled on gene expression data, and then predictions confirmed experimentally (an example is Brown et al., 2005). The genome of *Nicotiana tabacum* has has not yet been fully annotated, therefore function assignment through sequence homology identification to genes characterised in other species such as Arabidopsis, as well as comparison of expression patterns, remains a valuable contribution to research on tobacco and other *Solanaceae* family members.

The online resources to aid transcriptome analysis include Genevestigator (Hruz et al., 2008) and Genemania (Warde-Farley et al., 2010). Both of these tools link to microarray data from published research. Genevestigator allows visual display of expression data from different experiments as a single heatmap. The raw data is normalised using Bioconductor's RMA normalization (Gentleman et al., 2004) at a single experiment level. The data adjusted further with an inter-experiment correction, allowing comparison between gene expression under different conditions, in different tissue and at different developmental stages.

Genemania allows identification of genes that are co-expressed or co-localised to a set of input genes. Again, microarray data from published research is used. Links between genes are given a ‘weight’, measured by the GeneMANIA algorithm, which indicates the strength of the correlation (Warde-Farley et al., 2010).

Transcript levels can be measured for a small amount of genes using quantitative real time polymerase chain reaction (qRT-PCR). In this study qRT-PCR is used to quantify the effect of lignin or xylan down-regulation on the rest of the cell wall synthesis pathway at the transcript level. There is no consensus on the consequences of modification of one cell wall component or biosynthesis gene on the biosynthesis or deposition of other cell wall components. The analysis at the transcriptional level in tobacco will contribute to the understanding of the gene regulatory network involved and its implication to utilising plants with modified cell walls as an industrial resource.

1.7 Saccharification assays

1.7.1 Saccharification of cell wall material

As mentioned above, lignocellulosic biomass is an alternative source of fermentable sugars for bioethanol synthesis. This requires the extraction of sugars from the cell walls. Saccharification assays measure the amount of sugar released from cell wall or plant preparations by chemicals, microorganisms or enzymes. One of the earliest saccharification experiments was done by Saeman et al. (1945). It compared the yield of reducing sugar from fifteen different species of tree. Their results indicated that the hydrolysis products of aspen are the richest in fermentable reducing sugars. Since then, many saccharification studies have been carried out using different means of hydrolysis and different substrates. The effect of pretreatments that physically and chemically break down the cell wall matrix on sugar production have also been characterised using saccharification assays (Excoiffer et al*.,* 1991; Taherzadeh and Karimi, 2008; Hendriks and Zeeman, 2009).

In this project saccharification was used to compare differences in four different transgenic tobacco lines, each suppressed in one cell wall biosynthesis gene. Saccharification efficiency, which is indicative of the proportion of total sugars released from the cell wall material, has been compared between the four tobacco lines with modified secondary cell walls and their wildtypes and also used to test the effectiveness of pre-treating the cell wall material, and cell wall material from pectin modified Arabidopsis lines, with white rot fungi.

1.7.2 Complex sugars analysis

Gas chromatography-mass spectrometry (GC-MS) is used to separate and identify metabolites in a sample. GC separates the compounds using a gas phase such as helium and a ‘stationary’ liquid phase. The compounds in the sample are retained in the stationary phase for different lengths of time according to the partition constant of each compound in the system. The partition constant is defined by the equilibrium solute concentration in the liquid (stationary) phase and in the vapour phase, which depends in part on mass. The retention time increases with sample size (Castells, 2004). The molecules are passed separately into the ion source of the mass spectrometer, where they are ionised. The ions are again sorted into masses by the electromagnetic fields in the mass analyser. Throughout this project, GC-MS was used to identify monosaccharides released from the cell wall by various treatments.

1.8 Objectives

The principal aim of this study was to investigate the hypothesis that reducing lignin or xylan content improves the saccharification properties of lignocellulosic material. The starting material was four transgenic tobacco lines, previously generated and described in Section 2.3. As each line was down-regulated in a different cell wall synthesis gene, it was possible to assess the consequences for saccharification properties of changing carbon flux into the phenyl propanoid pathway, changing the final monolignol composition of lignin and of reducing the enzymes available at the final lignin polymerisation stage.

The second objective was to investigate the efficiency of fungal pretreatment on modified cell walls. White rot fungi metabolise lignin and improve saccharification efficiency (Hatakka, 1983; Zhang, 2007; Hwang, 2008) but their ability to pretreat material with reduced lignin or xylan content has never been tested.

Finally, it is important to understand the physical and molecular consequences to cell wall modification. The details of cell wall synthesis regulation are not well known, so another aim of the study was to establish what effect suppressing lignin or xylan genes has at the transcriptional level on the other cell wall components. In order to understand the differences in saccharification properties, the analysis was carried out on cell wall composition, vascular structure and transcriptional effects of cell wall modification.

2 Materials and Methods

2.1 Chemicals

Hydrochloric acid, sulphuric acid, acetic acid, methanol, chloroform, ethanol and acetone were purchased from VWR (Soulbury, Leighton Buzzard, UK). Malt extract agar was from Oxoid (Basingstoke, UK). Timentin and Murashige & Skoog MS Medium with Vitamins were both purchased from Melford (Chelsworth, Ipswich, UK). All other growth media, solvents, enzymes and chemicals were from Sigma-Aldrich (Poole, UK) unless otherwise stated.

2.2 Equipment

Visible light absorbance was measured with an Ultraspec 100 pro spectrophotometer which was supplied by Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Ultraviolet absorbance was read on a BioPhotometer spectrophotometer from Eppendorf (Histon, Cambridge, UK). Eppendorf also supplied the Centrifuge 5810R used in many experiments. Polymerase chain reaction (PCR) was performed using a Primus 25 thermocycler (Peqlab, Germany). Quantitative real time PCR (qRT-PCR) was performed using a Rotor-Gene Q real time machine (Qiagen, USA). An Excella E24R refrigerated Incubator shaker was used for incubations which required agitation, while a Memmert (Schwabach, Germany) incubator I was used for stationary incubations. RNA was quantified using a Nanodrop ND-6000 (NanoDrop Technologies, Delaware, USA).

Sugar analysis was performed on a Hewlett Packard 5970 series mass selective detector equipped with a c18 reverse phase column (250 x 4.6 mm 5 µm particle size, Phenomenex sphereclone) and a Hewlett Packard 5890 Series II gas chromatograph equipped with a Hewlett Packard 7673 Autosampler and a 25 x 0.22 mm i.d. HT5 column (both from Agilent technologies, Stockport, UK).

2.3 Plant materials

2.3.1 T-cyt tobacco cell cultures cDNA (EST) library

A xylogenic tobacco cell culture was derived from tobacco transformed with the *Tcyt* gene from *Agrobacterium* (Blee et al., 2001). The *Tcyt* gene enhances high endogenous levels of cytokinin, a growth-promoting hormone. The culture was originally set up for a proteomics investigation of tobacco cell walls (Blee et al., 2001). As described in Section 5.1**,** cDNA was synthesised from RNA isolated from the culture. A cDNA library and the derived ESTs were sequenced and deposited in NCBI (EH663598 – EH666265). The EST library has been key in identifying structural genes in the tobacco cell wall (Blee et al*.*, 2001) and for studying differences in their expression between transgenic and wildtype plants. These studies have been continued in this project, not only to improve knowledge of cell wall synthesis but to investigate unintended effects of transgenesis.

2.3.2 Cinnamoyl-CoA Reductase suppressed tobacco line (CCR)

O'Connell et al*.* (2002) generated the suppressed transgenic tobacco line used throughout this project. Suppression of *CCR* activity was by partial sense down-regulation. A fragment of the tobacco *CCR* gene was inserted into a XbaI-restricted pJR1Ri vector, which contains CaMV35S promoter and the 3' terminal end of the nopaline synthase gene (Smith et al., 1988). Tobacco leaf discs were transformed using *Agrobacterium* containing this vector.

O'Connell et al*.* (2002) determined that five of the transgenic lines had residual CCR activities of less than 50%. The line used in this investigation is *ccr86*, which had residual CCR activity of 1% and its Klason lignin content was 58% of a normal plant. Despite this, the height and vitality of the plants were not affected. The leaves of *ccr86* however were a darker green than normal, with a soft leaf texture and downward curl. A brown-orange colour in the xylem could also be seen. As mentioned in Section 1.3.2, plants in which *CCR* has been severely down-regulated suffer from collapsed vascular integrity, but 58% of wildtype level of lignin appears to be sufficient for the plant to be healthy. Monolignol composition was different in *CCR* down-regulated lines, meaning that kraft pulping, a process which turns wood chips into a wood pulp consisting mainly of cellulose, was improved even in the transgenic plants with a smaller reduction in CCR activity and lignin content. Line *ccr86* contained lignin with a syringyl/guaicyl ratio of 1.71 compared to 0.75 in the wildtype. O'Connell et al. (2002) also tested the transgenic *Nicotiana tabacum* v. Samsun (NVS) for cellulose extractability, and found *ccr86* to have the highest yield of cellulose after treatment.

2.3.3 Cinnamate-4-hydroxylase suppressed tobacco lines (C4H)

CINNAMATE-4-HYDROXYLASE (C4H) is the second enzyme of the phenylpropanoid pathway. Consequently it is a key component of the lignin biosynthetic pathway in addition to the synthesis of many flavonoids. C4H is a member of the cytochrome P450 (CYP) superfamily of enzymes. Most of the enzmes in this group catalyze redox reactions in which an organic substrate is oxidised by addition of an oxygen atom while the second oxygen atom in the oxygen molecule is reduced to water. C4H is a hydroxylase, and adds a hydroxyl group (-OH) to its substrate, cinnamic acid. The first C4H to be isolated and cloned was CYP73, which has many orthologues in different organisms. Orthologues with 60% sequence similarity to CYP73 are labelled Class II C4H. The *C4H*-downregulated transgenic transgenic *Nicotiana tabacum* v. Samsun (NVS) plants investigated in this project were generated and investigated by Blee et al. (2001). They cloned a French Bean (*Phaseolus vulgaris*) Class II *C4H* gene *CYP73A15* lacking the N-terminus sequence. The N-terminus is one of five domains which diverge from the original *CYP73* (Nedelkina et al.,1999). This clone was inserted into the binary vector pJR1Ri in sense and antisense orientation and in turn inserted into *Agrobacterium*. This was used to transform tobacco leaf discs.

The transformed plants showed a range of phenotypes. Both lignin levels and enzyme activity varied between plants and between young and old stems. The young stems showed more variability in C4H activity, which ranged from 110% to 10%. Conversely, no mature stems showed any detectable enzyme activity. All of the lines contained lignin. Analysis using the acetyl bromide method to quantify lignin content showed that all but two of the transgenic lines had reduced lignin content. The line used in this investigation is antisense line *c4h30*, which had an acetyl-bromide lignin content of 86% and *C4H* activity of 10% as compared to the wildtype.

2.3.4 Tobacco Peroxidase 60 suppressed tobacco lines (PRX)

The *PRX* down-regulated plants used in this study were produced by Blee et al. (2003), using *FRENCH BEAN PEROXIDASE 1* (*FBP1*) as a full length antisense probe to find tobacco peroxidases. *FPB1* is associated with vascular tissue development, accumulating at sites of secondary thickening (Smith et al., 1994) therefore was a good starting point for identification of lignification-active peroxidases. Of the three full length peroxidase gene sequences obtained by this method, one, *TP02*, had already been investigated (Lagrimini et al., 1987) and two were identical. The protein they code for is TP60. TP60 differs from other tobacco peroxidases in that it only has one single asparagine residue. Others have as many as four, which are the sites of N-linked glycosylation. The gene also has an ER signal peptide site. Antisense *TP60* probing

showed that the gene is more highly expressed in the stem than in the roots or the leaves, suggesting a role in xylem synthesis (Blee et al., 2003).

Antisense silencing (through *Agrobacterium*) of *TP60* using *TP60* and *FBP1* inserts in the antisense orientation resulted in 169 transgenic *Nicotiana tabacum* v. Samsun (NVS) lines. Blee et al. (2003) discovered that silencing this peroxidase results in up to 40-50% reduction in lignin content. Acetyl bromide determined lignin content in 1074, the line used in this investigation, was 77% of the wildtype. Although the vascular apparatus was modified no negative effects on viability were observed (Kavousi et al., 2010). Both guaiacyl and syringyl levels decreased linearly, so the overall ratio was not affected (Blee et al., 2003).

2.3.5 UDP-glucuronate decarboxylase suppressed tobacco lines (UXS)

In order to generated tobacco lines containing reduced xylan content, Bindschedler et al. (2007) produced 173 transgenic tobacco lines containing an insert for *UDP-GLUCURONATE 7* (*DCX7*). The insert was obtained by extracting RNA from a tobacco culture constitutively synthesising xylogenic tissue (synthesised by Blee et al., 2003), converting the mRNA to cDNA. The cDNA was then ampllified by PCR using degenerate primers designed using an alignment of four UDP-glucuronate decarboxylase genes from *Arabidopsis* and pea. The PCR product was used as probe to isolate four full length clones. Only one of these, UDPGlucADCX1, was shown to be a functional UDP-glucuronate decarboxylase by expression in *E. coli*. *DCX7* was the PCR product with the highest similarity to UDPGlucADCX1. *DCX7* was inserted in the sense and antisense orientation into the binary vector pBNPDV35S. The lines were tested for xylan content, lignin content, cellulose extractability and activity of DCX. The line used in this experiment, *10643 52,* has 22% reduction in xylan content as compared to the wildtype, *Nicotiana tabacum* cultivar K326. None of the lines had unusual lignin content (Bindschedler et al., 2007), despite increased pulpability characteristics in the antisense lines. The DCX down-regulated lines also showed no evidence of increased cellulose or lignin levels in order to compensating for the reduced xylan content. In the paper describing the generation and first analysis of this line (Bindschedler et al., 2007), UDP-GLUCURONATE DECARBOXYLASE is abbreviated to DCX. However it is more common to abbreviate it to UXS (Pattathil et al., 2005; Suzuki et al., 2003; Harper et al., 2002) so this is how the line is referred to in the rest of this document.

Table 3 Summary of transgenic tobacco plants

Line name used in text	Transgenesis	Wildtype variety	Enzyme activity compared to wildtype	Target cell wall component level compared to wildtype *
ccr	Partial sense down-regulation of *CINNAMOYL CO-A REDUCTASE*	NVS	1% **	58% (Klason lignin)
c4h	Antisense down-regulation of *CINNAMATE 4-HYDROXYLASE*	NVS	10% ***	86% (acetyl bromide lignin)
prx	Antisense down-regulation of *TOBACCO PEROXIDASE 60*	NVS	Not determined	77 % (acetyl bromide lignin)
uxs	Antisense down-regulation of *UDP-GLUCURONATE DECARBOXYLASE*	K326	83% ****	78% (Xylose content)

* Cook et al., 2011
** O'Connell et al., 2002
*** Blee et al., 2001
**** Bindschedler et al., 2007

2.3.6 Arabidopsis lines with modified pectin: Polygalacturonase over-expressor (pg57) and pectin methylesterase inhibitor over-expressor (pmei2)

Capodicasa et al. (2004) described Arabidopsis lines expressing a polygalacturonase II gene from *Aspergillus niger*. Pectin is hydrolysed by plant pathogenic fungi by polygalacturonase (PG) enzymes in order to degrade the cell wall and access the cells (Buchanan et al., 2000). When expressed *in planta*, *Aspergillus niger PGII* causes a 25% reduction in uronic acid content, and reduces the lebels of deesterified homogalacturonan. These *pg57* lines were dwarfed, and contained 25% less uronic acid than the wildtype line (Capodicasa et al., 2004). The *PGII* over-expression improves saccharification efficiency twofold (Lionetti et al., 2010).

Lionetti et al. (2007) described Arabidopsis lines over-expressing an endogenous pectin methylesterase inhibitor (*pmei*). Pectin methylesterases (PME) demethylesterify homogalacturonan into a form that is incorporated into the cell wall and becomes integral to its structure. Demethylesterified homogalacturonan can form Ca^{2+} bonds

with other pectin molecules (Mohnen, 2008; Pelloux et al., 2007). PME inhibitors (PMEIs) prevent PME activity, therefore limiting cross-linking in the primary cell wall. *pmei* lines have 16% more methylesterified homogalacturonan than wildtype and are resistant to *Botrytis cinerea* (Lionetti et al., 2007). *PMEI* overexpressers also have a saccharification efficiency of up to 50% more than wildtype (Lionetti et al., 2010). The same transgenesis also improved saccharification efficiency of tobacco leaves and wheat stem material. The line used in this study is *A. thaliana* line *pmei2*. The pectin methylesterase inhibition caused the degree of pectin methylesterification in *pmei2* to be increased by 16%. It also causes resistance to *Botrytis cinerea*.

2.4 General plant techniques

2.4.1 Growth and maintenance of *Nicotiana tabacum* plants

Wildtype tobacco line *Nicotiana tabacum v. Samsun* (NVS) with lines down-regulated in *C4H (c4h), PRX (prx)* and *CCR (ccr)* (Section 2.3) as well as a *UXS*-down-regulated line (*UXS)* with its wildtype *Nicotiana tabacum* K326 (K326) were propagated from cuttings and grown on Levington M3 Pot and Bedding Compost High Nutrient (The Scotts Company LLC) in a glasshouse. Supplementary lighting allowed a photoperiod of 16 hours. The photo-synthetically active radiation at crop height is very variable as the plants were outside and not in a controlled environment. The light intensity range was between 73 and 1380 µmol m^{-2} s^{-1}. This was dependent on the season and the time of day. High pressure sodium lamps at 400 watts provided supplementary lighting during the winter months. The temperature was between 20°C and 25°C, as the temperature fell at night. Lines *c4h, ccr, prx* and *uxs* have been described previously (Blee et al., 2001; O'Connell et al., 2002; Blee et al., 2003; Bindschedler et al., 2007 respectively).

2.4.2 Growth and maintenance of *Arabidopsis thaliana* plants

Arabidopsis thaliana ecotype Columbia (Col-0) and transgenic *Arabidopsis* seeds were germinated asceptically on 0.8% agar Murashige and Skoog (MS) medium supplemented with 5% sucrose and 0.25% 2-(N-morpholino)ethanesulfonic acid, following 24 hour stratification at 4°C. The seedlings were transferred to soil after 15 days and grown in a climate controlled environment at 23°C. Supplementary lighting allowed a photoperiod of 16 hours. Light intensity was 120 µmol m^{-2} s^{-1}. The *RAP2.12*

insertion lines SALK_152421 and SAIL_1215 were purchased from the European Arabidopsis Stock Centre NASC (Nottingham, UK).

2.4.3 Extraction of cell wall material from fresh plant tissue

The generation of acetone insoluble material was adapted from Robertson et al. (1999). Stem sections were cut to approximately 5 mm in length and homogenised in 14 g batches in 120 ml of pre-chilled acetone for three minutes. The acetone was collected in falcon tubes and the remaining stem sections sliced with a razor into pieces roughly 1-2 mm in width and length. These were again homogenised in 100 ml of glacial acetone for four minutes. The acetone and insoluble material was collected in falcon tubes. The insoluble solids were collected by centrifugation at 3500 rpm for ten minutes.

The pellets were air dried and ground under liquid nitrogen. The ground samples were treated according to Robertson et al. (1999). They were washed three times in 80% acetone to remove sugars, twice in methanol/chloroform to dissolve lipids and again in glacial acetone. The pellet was deproteinised in phenol/acetone/water (2:1:1) and washed three more times in 80% acetone. All the washes were carried out using 5x volume of the pelleted material followed by centrifugation for ten minutes at 2205 x *g* (3500 rpm). The final pellet was air dried.

The acetone insoluble material was de-starched by addition of amylase (α-amylase from porcine pancreas; SigmaAldrich, Dorset, UK) and amyloglucosidase (Amyloglucosidase from *Aspergillus niger*; SigmaAldrich, Dorset, UK), both 5U in 10 ml of 1M sodium acetate. The biomass was incubated in the buffer at 60 °C for ten minutes before overnight incubation at 37 °C in an incubator shaking at 100 rpm. The pellet was washed twice with water, followed by a final wash with acetone before air drying.

2.5 General molecular biology techniques

2.5.1 RNA extraction and cDNA synthesis

Total RNA was extracted from tobacco xylem tissue, *Arabidposis thaliana* leaf tissue or *Phanerochaete chrysosporium* mycelia. The biomass was ground under liquid nitrogen, with the RNeasy Minikit (Qiagen, UK) following the manufacturer's instructions and tested using a Nanodrop ND-6000. RNA then reverse transcribed to cDNA using the QuantiTect Reverse Transcription kit (Qiagen, UK), according to the manufacturers instructions.

2.5.2 Genomic DNA extraction

The DNA extraction method was adapted from Edwards et al. (1991). Plant tissue was hand ground with a mortar and pestle under liquid nitrogen. To around 20 mg of ground tissue, 400 µl of extraction buffer was added and the mixture homogenised with a Micropestle (Eppendorf, Histon, Cambridge, UK). The extraction buffer comprised Edwards Solution (200 mM Tris-HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA, and 0.5% SDS) diluted ten-fold with TE (10 mM Tris-HCl (pH 7.5), 1 mM EDTA) buffer. The homogenate was centrifuged at 9477 x *g* (13000 rpm) for two minutes and the supernatant transferred to a new Eppendorf tube. One volume of ice cold isopropanol was added and the mixture left on ice for ten minutes then centrifuged at 13 000 rpm for five minutes. The supernatant was discarded and the pellet was dried under a laminar flow cabinet for ten minutes. Following drying, the pellet was re-suspended in 100 µl of sterile distilled water and stored at -20 °C.

2.5.3 Polymerase chain reaction

A standard PCR reaction tube contained the following:

Reagent	Volume	Final concentration
5x Green GoTaq Reaction Buffer *	10 µl	1X
Magnesium chloride (25mM)	4 µl	2 mM
dNTPs (10mM)	1 µl	0.2 mM each dNTP
Upstream primer	1 µl	1 µM
Downstream primer	1 µl	1 µM
Template DNA/cDNA	0.5 µl	0.01 µg/µl
GoTaq DNA Polymerase *	0.25 µl	1.25u/50µl
Sterile distilled water	32.25	
*Promega, Southampton, UK		

The PCR conditions were as follows: two minutes at 94°C followed by 25-35 cycles of denaturing at 94°C for 40 sec, annealing at 56-60°C for 60 sec and extending for 40 sec at 72°C. After the correct number of cycles there was a final extension period of ten minutes at 72°C before resting at 14°C. The products were analysed by gel electrophoresis. The samples were run under constant voltage on a 1% agarose gel containing 1X SYBR Safe DNA gel stain (Invitrogen, USA) and were visualised under UV light.

Table 4 List of primers used

Gene	Name	Accession number	Forward (5' to 3') sequence	Reverse (3' to 5') sequence	Size of amplicon (bp)
Tobacco qRT-PCR primers					
Cell wall synthesis genes					
Phenylalanine ammonia lyase	PAL	D17467	GCAAACAGCTCAA TCTTCCA	TCGACTTCTTTTG GCAACAC	74
Secretory peroxidase	PRX	AF149251	CTTGCCAACAAGC TCCACTA	CAAAGGAAGGGGA AAAGTGA	76
Cinnamoyl Co-A reductase	CCR	AY149609	TGTGTCTTCTGTT GCTGCTG	ATTCACTGTCCGA CCAACAA	78
Cinnamoyl alcohol dehydrogenase	CAD	EH664196	TGGAACATCTTGG TGCAGAT	ATGGCCAACAGGG ACAGTAT	107
Catechol O-methyltransferase	COMT	EH663855	ACATAACCCAGGA GGCAAAG	TTCCATGACCCAA GTGTTGT	114
Caffeoyl Co-A methyl O-transferase	coCOMT	EH665253	ATTTTCGTGGATG CTGACAA	GTCGTAGCCAATC ACACCAC	90
Cinnamate-4-hydroxylase	C4H	EH664914	AGCAATGCTCTGA AATGTGC	CCTCAGTTGATCT CCCCTTC	67
P-coumarate-3-hydroxylase	C3H	EH663728	AGCAGTGGCCTTT AACAACA	GTCACCATCACAC TTCAAAG	75
Sucrose synthase	SuSy	EH664745	GAAGCAAGGACAC TGTTGGA	ATACAATCCAGGC ATCGTGA	62
Cellulose	CES3A	EH663724	TGGAATTGATGAA	CAACCCTTGGAAG	90

synthase A3			TGGTGGA	ACCTAGC	
UDP-glucuronate decarboxylase	UXS	EH663981	AAAACCACCACCA GAACCAT	CAATAAATCCAGC ACCACCA	93
Cellulose synthase like D	CslD	EH665280	GGAAAGGAACTTG GAAGTGG	AATCTGCACAATC CCACGTA	80
UDP-D-glucuronate 4-epimerase	UG4E	EH664843	GGGGTCGTATTTG TGTTTCC	TGTTTCTCCCAAT GATGACC	88
UDP-glucose dehyrogenase	UGD	EH663670	AATGAGTCCAACA ACCGTGA	TCCTTTGTTGCTG TGTAGGC	63
ADH-like UDP-glucose dehydrogenase	ADH	AY619949	AATGCCATGTCAG CTCTTTG	AATGCCATGTCAG CTCTTTG	60
Transcription factors with xylem specific expression					
Related to APETALA2	RAP2	EH665541	CGAGGTGTGAAGG TTGAGAA	CCACGGTCTCTGC CTTATTC	82
Short vegetative phase	SVP	EH665729	CCACGGTCTCTGC CTTATTC	GGTCAATCCAGCT TCCAGAG	85
Argenine/serine rich zinc knuckle containing protein	RSZ33	EH663821	TGGAGGACGTCTT TAGCAGA	CATCAGCATCTCG AGGATCA	98
Revoluta	REV	EH663642	GCTGTCGATATGC AGAGGAA	CAGCAGTTCCTGT AGCCTTG	62
Arabidopsis RT-PCR primers					
Secretory peroxidase	AtPRX	AT4G21960	CGATACCGATGGA TTTGAAC	TCTCTTGGCTCCG TAACATC	591
Cinnamoyl Co-A reductase	AtCCR	At2g33590	GAAGCGAATGTTA AGCGTGT	CACGCACATCTAC CAAATGA	533
Cinnamate-4-hydroxylase	AtC4H	At2g30490	GGCACATTTCAAT CCTTCAC	TCATGACGGTTCC TTTCTTC	525
Cellulose synthase A3	AtCESA 3	AT5G05170	GTCCAACAGGATC CACAATC	GAGGATGGCTTAG CTGATGA	515
UDP-glucuronate decarboxylase	AtUXS	AT3G46440	CGAGGTTGAGGTA AGCAAAA	TGGATTCATTGGT TCTCACC	586
Genotyping RAP2.12 insertion lines					

Related to APETALA2	AtRAP2.12	AT1G53910	TTCGTTTTCACTTCGACTCC	TCCAAGCCAGATTCTAGCAC	811
SAIL LB_1/ RAP2.12_R			GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC	TCCAAGCCAGATTCTAGCAC	1030
SAIL LB_1/ RAP2.12_L			GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC	TTCGTTTTCACTTCGACTCC	647
SALK LBa1/ RAP2.12_R#2			TGGTTCACGTAGTGGGCCATCG	GCTTGGACTTCTTGCCATAG	276
SALK LBa1/ RAP2.12_L			TGGTTCACGTAGTGGGCCATCG	TTCGTTTTCACTTCGACTCC	1141
***Phanerochaete chrysosporium* RT-PCR primers**					
a-tubulin1	a-tub	AADS01000121	GCTTGGACTTCTTGCCATAG	TGGCTTCAGCACTTTCTTCT	411
18S RNA	PC18S	GU966518	TGGCTCATCCACTCTTCAAC	AAGCGATCCGTTACACTCAC	415

2.5.4 Genotyping *A. thaliana* insertion lines

Genomic DNA was extracted from leaves one and two when the plants were at the four-leaf stage. The DNA was subjected to polymerase chain reaction (PCR) with the primer sets shown in Table 4. Primers from the left border of the insertion (SAIL LB or SALK LBa1; Sessions et al., 2002 and Alonso et al., 2003 respectively) were paired with primers designed upstream and downstream of the insertion in the RAP2.12 gene (RAP2.12_L and RAP2.12_R). Using both upstream and downstream primers with the left border primer ensured amplification of the insertion if it was in the reverse orientation as well as the forward orientation. As the SALK insertion was further towards the 3 prime end of the coding sequence than RAP2.12_R, a second downstream primer, RAP2.12_R#2, was designed so that the SALK insertion could be amplified. Following PCR, the products were analysed by gel electrophoresis.

2.6 Microscopy analysis of cell walls

2.6.1 Tobacco cross sections

A razor blade was used to cut four freehand sections of 1-2 mm thickness of tobacco stem from the midpoint of internodes 3 of one plant from each of the tobacco lines. All the plants were over six months old and had 15-20 internodes.

2.6.2 Arabidopsis cross sections

Five weeks after sowing, Arabidopsis stems were harvested and the third internode cut out. Slightly cooled 3% agarose gel was poured over the third internode. When the gel was set, a razor blade was used to cut four freehand sections 0.5 mm in thickness.

2.6.3 Phloroglucinol Staining

Phloroglucinol (1,3,5-benzenetriol; $C_6H_6O_3$) stains lignin. In 12 well microtitre plates, 1 ml of 0.1% phloroglucinol in 70% ethanol was added to the stem cross sections. After an incubation period of 15 minutes this was removed, and 1ml of 6M hydrochloric acid was added. Photographs were taken of the stained cross-sections. They were then examined through a Nikon Optiphot-2 photomicroscope.

2.6.4 Toluidene Blue Staining

Toluidene blue ((7-amino-8-methyl-phenothiazin-3-ylidene)-dimethyl-ammonium; $C_{15}H_{16}N_3S^+$) is a stain that detects cellulose fibres and lignin. A 1% (w/v) toluidene blue stock was made with 70% ethanol. This was diluted 1:10 with 1% sodium chloride. The working stock was added to the stem cross sections in a 12 well microtitre plate. After 2 minutes, the toluidene blue was removed and the stem sections washed five times with sterile water. Photographs were taken of the stained cross-sections. They were then examined through a Nikon Optiphot-2 photomicroscope.

2.7 Annotation and analysis of cDNA library from xylogenic tobacco cell culture

The ESTs obtained from the xylogenic tobacco culture (Blee et al., 2001) were aligned and annotated with genes from the NCBI BLASTn database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), where the top annotated hit was used with preference for *Solanaceae* genes. They were also aligned with genes from the TAIR

database of Arabidopsis genes. Arabidopsis genes are more suitable for further investigation including expression and localisation analysis as there are online resources for Arabidopsis but not for the incompletely annotated tobacco genome. In some cases the closest alignments from *Solanaceae* and Arabidopsis were not the same. In these cases, if the *Solanacae* gene encoded a known cell wall related gene, its closest Arabidopsis homologue was found and used for further analysis. Online resources used to investigate gene expression profiles and co-expression connections were Genevestigator (Hruz et al., 2008), Genemania (Warde-Farley et al., 2010) and EFP browser (Winter et al., 2007).

2.8 Quantifying mRNA transcripts related to transcription factors and cell wall synthesis using quantitative real-time PCR

2.8.1 Primer design and synthesis

Primers were designed using Primer 3 tool (Massachusetts Institute of Technology, Massachusetts) using the default settings with the following exceptions: the primers are 20 or 21 base pairs long; qRT-PCR primers produced fragments of DNA 67 – 98 base pairs in length; and PCR primers had a product of between 400 and 600 base pairs. They have primer melting temperatures (Tm) of 57°C – 59°C and the C:G ratios are around 50%. The primers were synthesised by Eurofins MWG Operon (London). The primers are listed in Table 4.

2.8.2 quantitative Real Time Polymerase Chain Reaction

The 2x Sensimix dU SYBR Green kit (Quantace, UK) was used in qRT-PCR preparation. The reaction was carried out in a Rotor-Gene Q (Quiagen, UK) machine. The conditions used were as follows: ten minutes at 95°C followed by 40 cycles of denaturing at 95°C for 10 seconds, annealing at 58°C for 15 seconds and extending for 20 seconds at 72°C. The final cycle consisted of a denaturing period of ten seconds at 95°C as previously but shortened denaturing and annealing times of five seconds each. In order to detect amplification of the fragments corresponding to the transcription factor genes *SVP, REV, RSZ33* and *RAP2,* five extra cycles were added.

2.9 Fractionation and quantification of cell wall components

The protocol was adapted from Robertson et al. (1999). 0.05 g of cell wall extract was sonicated in 5 ml of 50mM CDTA/50mM acetate buffer and stirred for 15 hours at room temperature. The mixture was centrifuged for 10 minutes at 9477 x *g* (13000 rpm) and the supernatant removed. The pellet was washed with a further 2 ml of 50 mM CDTA/50 mM acetate buffer, centrifuged and the supernatants combined. The combined supernatant was filtered from Whatman GF/A paper and dialysed against deionised water for seven days with two changes of water per day. The pellet from the 50 mM CDTA/50mM acetate buffer extraction was sonicated in 5 ml 50 mM $NaCO_3$/0.1 M $NaBH_4$. The mixture was stirred for 15 hours at 4°C and for a further two hours at room temperature. The mixture was centrifuged at 9477 x *g* (13000 rpm) for 10 minutes. The pellet was washed in 2 ml of 5 ml 50 mM $NaCO_3$/0.1 M $NaBH_4$ and the supernatants were pooled, neutralised with glacial acetic acid and filtered as before. The extract was then dialysed against dionised water for five days with two changes of water per day. The pellet from the previous extraction was sonicated in 5 ml 4 M KOH/0.1 M $NaBH_4$ and the tube was flooded with nitrogen. The mixture was incubated at room temperature for two hours on a rotary shaker. The mixture was centrifuged and washed as previously. Finally 5 ml of water was added to the pellet. Both the 5 ml 4 M KOH/0.1 M $NaBH_4$ extract and the water mixture were neutralised with acetic acid. The 4 M KOH/0.1 M $NaBH_4$ solution was filtered and both mixtures were dialysed for five days against deionised water with 2 changes of water per day. When the dialysis period was over, all extracts were freeze dried and weighed. After weighing, the lignocellulose fraction was subjected to total carbohydrate analysis (see Section 2.13) in order to ascertain cellulose content.

2.10 Saccharification Assays

The saccharification assay protocol was adapted from NREL/TP-510-42629 (Selig et al., 2008). In order to kill any microorganisms that might have survived the cell wall extraction and to denature any residual enzymes, 0.05 g of cell wall extract was heated at 100°C for ten minutes in 2 ml Eppendorf tubes. The samples were subsequently cooled on ice.

Each reaction tube consisted of 10 mg AIM soaked in 2 units each of cellulase from Aspergillus, Cellulase from Tricoderma, Driselase and Maccerase in 0.05M Sodium citrate buffer (pH4), with 0.4% antibiotic (Kanamycin:tetracycline:gentamycin 4:3:1).

One unit will liberate 1.0 µmole of glucose from cellulose in one hour at pH 5.0 at 37°C with a 2 hour total incubation time (SigmaAldrich, 2009). Driselase is a cell wall degrading enzyme preparation from Basidiomycetes sp (SigmaAldrich, 2009). The final concentration of each antibiotic was: kanamycin 100 µg/ml; tetracycline 15 µg/ml; and gentamicin 25 µg/ml. The samples were incubated at 50°C, shaking at 200 rpm, for 72 hours.

The volumes used are given in Table 5. The experimental plan, including controls, is given in Figure 15. The controls were the enzyme control in which the full reaction mix, including enzymes, were incubated with no AIM, and the AIM control in which the AIM was incubated with the buffer and antibiotics but no enzymes. Following incubation, the samples were centrifuged at 9477 x *g* (13000 rpm) and the supernatant was analysed for reducing sugars using the phenol sulphuric acid assay (Fry, 1988).

Table 5 Volumes of reagents in saccharification assay

	Reaction	Substrate control	Enzyme Control
AIM	0.05 g	0.05 g	0
Enzyme cocktail	100 µl	0	100 µl
0.1M sodium citrate buffer pH 4.8	500 µl	500 µl	500 µl
Antibiotic cocktail	4 µl	4 µl	4 µl
Sterile distilled water	346 µl	446 µl	396 µl

2.10.1 Phenol sulphuric acid assay of saccharification supernatant to detect free sugars

After saccharification, the samples were spun in a centrifuge at 9477 x *g* (13 000 rpm) and the supernatant analysed to determine the total sugar content using the phenol sulphuric acid assay protocol described by Fry (1988). For this, the samples were diluted x100 into 400 µl double autoclaved water in a 15 ml glass tube. 10 µl of 80% phenol was added and the samples were mixed by vortexing. Then 1 ml of concentrated sulphuric acid was pipetted directly into the mixture drop by drop and mixed by vortexing. The absorbance of the mixture was recorded at A_{480} using a Ultraspec 100 pro spectrophotometer (Amersham Biosciences). The absorbance reading from the enzyme control was subtracted from the absorbance reading of each saccharified sample. The mass of glucose equivalents measured in the phenol sulphuric assay were calculated from the absorbance, which now took into account sugar released from the enzyme background, using a glucose standard curve. This

figure was multiplied to obtain the mass of glucose equivalents in the supernatant of the saccharification assay. This figure was then converted to µMol by being divided by the molecular mass of glucose (180 g/mol) and divided by the mg of AIM saccharified to obtain the µMol glucose equivalents released per mg AIM. The formula was as follows:

$$\mu\text{mol sugar/mg AIM} = \frac{\mu\text{g} / 180}{\text{mg AIM}}$$

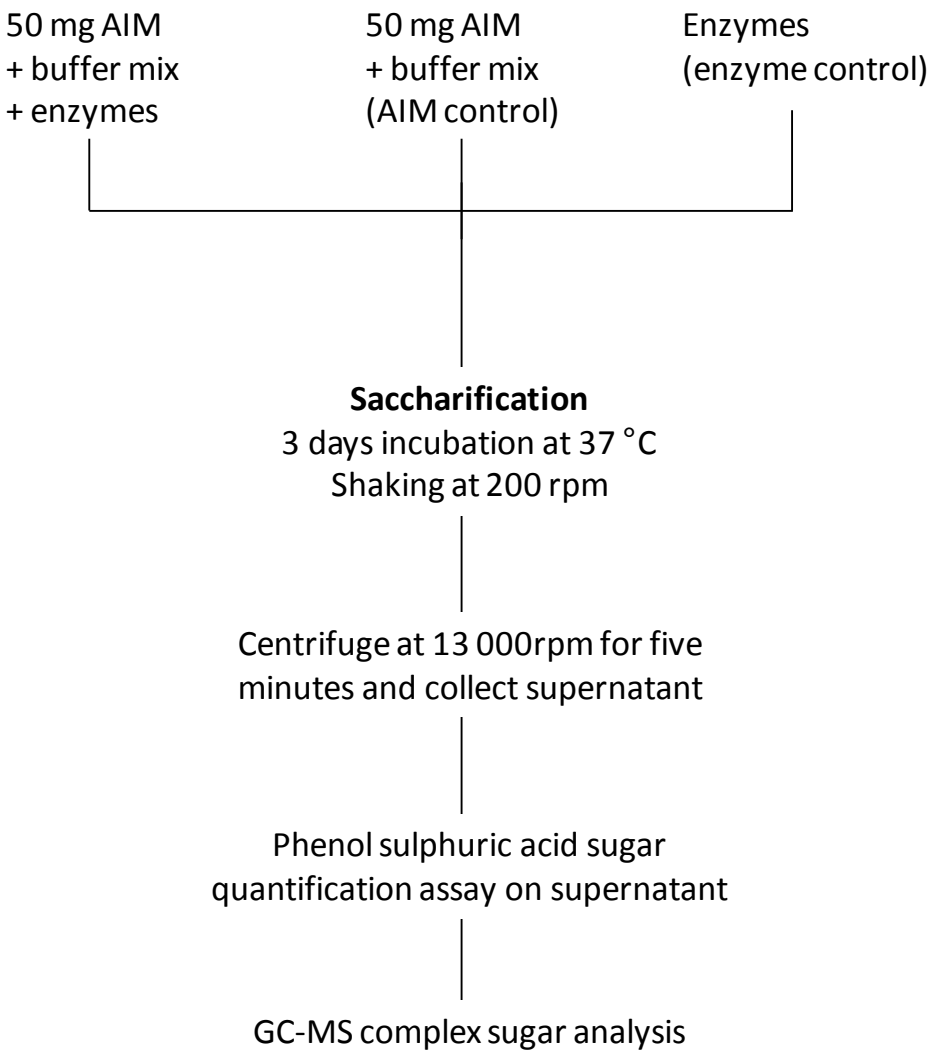


Figure 15 Schematic diagram of the protocol used for testing the saccharification properties of the tobacco acetone insoluble cell wall material (AIM).

50 mg aliquots of AIM are treated with an enzyme mixture. Incubation is at 50 °C for 72 hours. Two controls are incubated alongside the saccharified AIM: an enzyme control, containing no AIM substrate, and an AIM control which contains no enzymes. After 72 hours the solid material is pelleted and the sugar content of the supernatant is quantified by the phenol sulphuric acid assay.

2.11 Pretreatment of tobacco cell wall material with *Phanerochaete chrysosporium*

2.11.1 Culture of *Phanerochaete chrysosporium*

Phanerochaete chrysosporium strain 24725 was grown on modified malt agar plates (malt extract 1.6%, glucose 1%, yeast extract 0.2%, asparagine 0.1%, potassium hydrogen orthophosphate 0.2%, magnesium sulphate 0.1%, agar 1.5%). It was sub-cultured by taking a slab of sporulating fungi on agar using a sterile spatula which had been soaked in ethanol and flamed and placing it on a fresh malt agar plate.

2.11.2 Fungal pretreatment of acetone insoluble cell wall material (AIM)

The fungal pretreatment method is shown in Figure 16. Experiments were done in triplicate, with a water control and a culture medium control (culture medium excluding fungus). 9 x 10 mg of acetone insoluble material (AIM), which was to be saccharified after pretreatment or incubation with the controls, was weighed into 2 ml Eppendorf tubes and heated on the dry water bath (Techne Dri-block DB-1; *Bibb*y *Scientific* Staffordshire, UK) at 100°C for 15 minutes. At the same time, 18 x 1 mg AIM was treated in the same way. The 18 samples were used as follows: nine samples to determine lignin content after pretreatment or control incubations, and nine samples to quantify total carbohydrate content after pretreatment or control incubations.

The final composition of the culture medium (adapted from Tien and Kirk, 1988) was: potassium dihydrogen orthophosphate (KH_2PO_4) 14.7 mM; magnesium sulphate ($MgSO_4$) 8.75 mM; calcium chloride ($CaCl_2$) 0.901 mM; manganese sulphate ($MnSO_4(H_2O)$ 0.527 mM; sodium chloride (NaCl) 2.73mM; iron sulphate ($Fe_2(SO_4)_3$ 0.105 mM; zinc sulphate ($ZnSO_4$) 0.099 mM; glucose ($C_6H_{12}O_6$) 55.6 mM; thiamin ($C_{12}H_{17}ClN_4OS$) 0.00332 mM; veratryl alcohol ($C_9H_{12}O_3$) 0.4 mM.

Culture medium was put together as follows (adapted from Tien and Kirk, 1988): 100ml Basal medium (potassium dihydrogen orthophosphate 147 mM; magnesium sulphate 41.5mM; calcium chloride 9.01mM; 100ml trace elements solution), 60ml trace elements solution (magnesium sulphate 24.9 mM; manganese sulphate 3.31 mM; sodium chloride 17.1 mM; iron sulphate 0.658 mM; zinc sulphate 0.619 mM), 100 ml 10% glucose, 10 ml thiamin (0.332 mM), 100ml veratryl alcohol (4 mM), sterile distilled water up to 1000 ml. Basal medium, trace elements solution, glucose and water were autoclaved. The other elements were filter sterilised and the incubation medium made under sterile conditions.

The culture medium was inoculated with a 5 mm x 5 mm slab of fungal mycelium, equivalent to 100 spores per ml medium. The slab was removed from a modified malt agar plate that had been growing at 37°C for 24 hours and homogenized into 50 ml culture medium. 2 ml of inoculated culture medium was added to 10 mg AIM in a 15ml falcon tube. The tubes were laid on their sides and incubated at 37°C for the ten day incubation period. 2 ml of uninoculated culture medium and 2 ml of water were added to 3 x 10 mg AIM. The 1 mg aliquots of AIM were treated in the same way with 60 µl culture media, medium or water. After 10 days, the samples were centrifuged at 2205 x *g* (3500rpm) for 5 minutes. The supernatant was removed and the pellet, consisting of AIM and mycelium, washed twice with 80% acetone. The pellet was removed from the falcon tube and transferred to a 2 ml Eppendorf tube, dried at 40°C on a dry bath and then heated to 100°C for 15 minutes to kill the fungus.

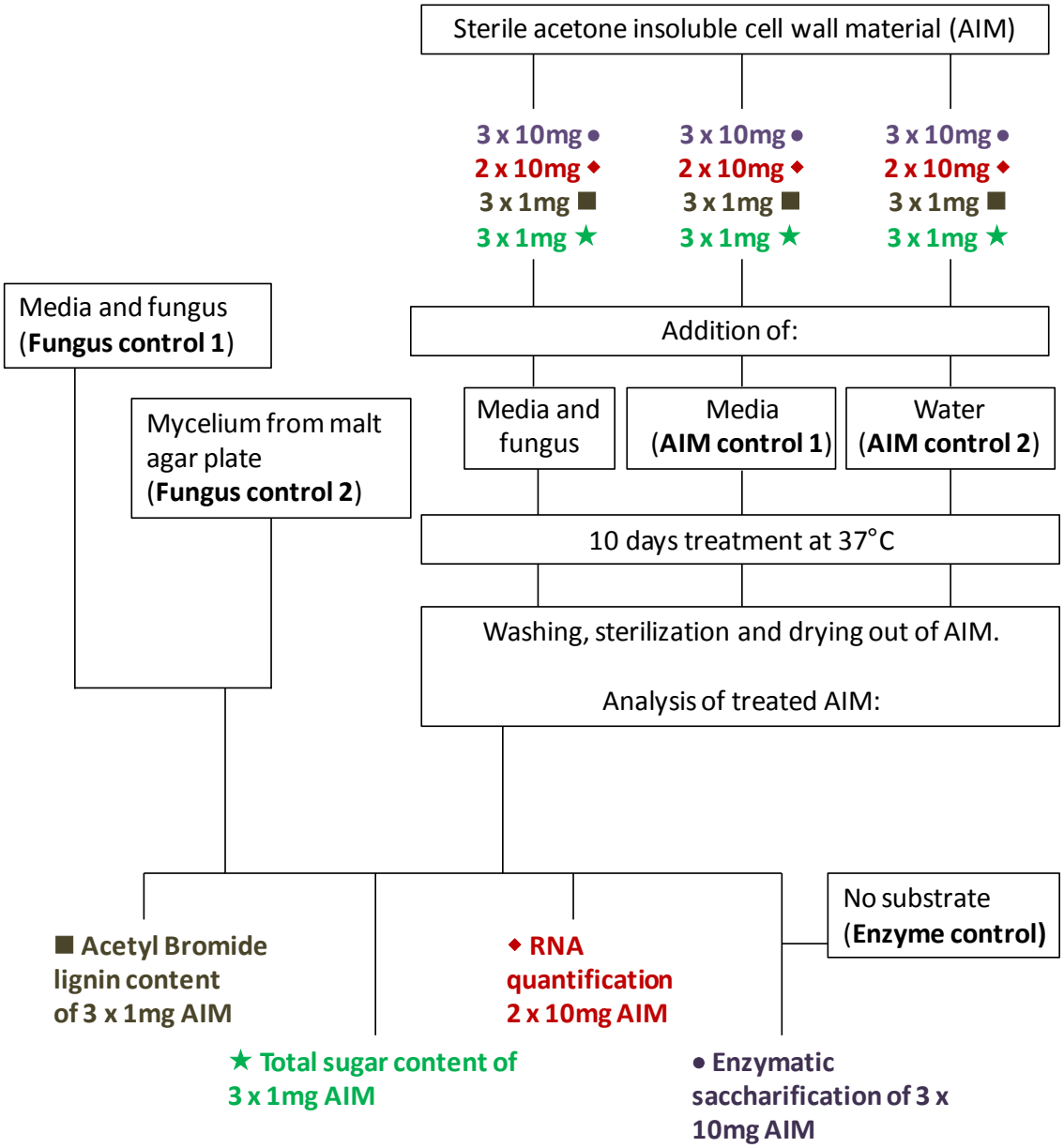


Figure 16 Schematic of protocol for pretreating acetone insoluble cell wall material (AIM)

Aliquots of AIM are treated with incubated culture media, control culture media or water. After washing, drying and sterilization the aliquots are analysed for acetyl bromide lignin content (■), total sugar content (★), fungal RNA (◆) and saccharification properties (●). An enzyme control, with no substrate, is saccharified. As there is very little fungal growth in inoculated culture media with no AIM substrate, aliquots of fungal mycelieum from an agar plate are analysed as well.

2.11.3 Fungal pretreatment of fresh plant biomass

In order to assess the effectiveness of *Phanerocahete chrysosporium* pretreatment on fresh plant biomass, *Arabidopsis thaliana* stems were pretreated with the same culture media as described in section 2.11.2. Internodes 1-5 of the primary stems from three 8-week old *Arabidopsis thaliana* ecotype Columbia (Col-0) were excised and cut into sections approximately 5 mm in length. The stem sections were collected into groups, each group weighing 25 mg in total. Each 25 mg sample was placed into a well in a 12-well tissue culture plate and sterilised with 2 ml 2% bleach (Parazone thick bleach) for two minutes, The samples were washed three more times in sterile water under sterile conditions, and left to soak in sterile water for one hour before one final wash. 1 ml of inoculated culture medium, prepared as described in Section 2.11.2, was added to half of the plant samples. 1 ml of water was added to the remaining samples. The samples were incubated in a stationary incubator at 37°C for one week. The following week the stems were removed from the wells. The stem sections that had been pretreated were removed from the mycelial mat. All the samples were put into sterile 1.5 ml Eppendorf tubes and washed once with 2% bleach, followed by three washes with water.

The stem sections were subjected to saccharification in the Eppendorf tubes using a method adapted from Lionetti et al. (2010). The enzyme solution was 1% Celluclast and 0.4% antibiotic cocktail in 50 mM sodium acetate buffer (pH5.5). The antibiotic cocktail was as described in Section 1.10.1. The stem sections incubated at 37°C in an incubator-shaker at 180rpm for 24 hours. Following saccharification the tubes were centrifuged at 5590 x *g* (10000 rpm) for five minutes and the supernatant analysed to determine sugar content using the phenol-sulphuric acid assay as described in Section 2.10.1.

2.12 Lignin quantification

The acetyl bromide method to quantify lignin content was adapted from described by Foster et al. (2010). 1-1.5 mg of acetone insoluble material (AIM) was weighed into screw top 10 ml glass tubes and the sides of the tubes washed with acetone ($(CH_3)_2CO$) to ensure all the AIM was collected at the bottom of the tube. The samples were heated at 50°C to evaporate the acetone. Once the AIM was dry, 100 µl of freshly made acetyl bromide solution (25% v/v acetyl bromide (C_2H_3BrO) in glacial acetic acid (CH_3COOH)) was gently added to the sample and the tubes were heated at 50°C for three hours, mixed by vortexing every 15 minutes throughout the final hour. The tubes

were transferred briefly to ice to cool to room temperature. Next, 400 µl of 2M sodium hydroxide (NaOH) and 70 µl of freshly prepared 0.5M hydroxylamine hydrochloride ($HONH_2 \cdot HCl$) were added to the sample and the tube was gently vortexed. The assay volume was made up to 2 ml total volume with glacial acetic acid and mixed. Finally, the absorbance of the sample was detected at A280.

2.13 Total carbohydrate quantification

In order to have three technical replicates for each line, three times 1 mg of AIM was weighed into a screw-cap 2 ml tube. To each sample, 40 µl of 72% (v/v) sulphuric acid (H_2SO_4) was added, the cap screwed on tightly and the samples incubated on a dry water bath at 37°C for 90 minutes. 756 µl water was added to each sample to make 4% sulphuric acid. The tubes were vortexed, centrifuged briefly and heated at 120°C for 90 minutes on the dry water bath, with the caps loosely screwed on. The samples were then removed from the heat and cooled to room temperature. The tubes were centrifuged at 5590 x *g* (10000 rpm) for five minutes. The sugar in the supernatant was quantified using the phenol-sulphuric acid assay as described in Section 2.10.2.

2.14 Analysis of sugars with gas chromatography mass spectrometry (GC-MS)

2.14.1 Preparation of sugars for GC-MS

In order to determine monosaccharide content of the cell wall fractions and of the sugars released from cell walls by saccharification, the samples were analysed using GC-MS. The sugar in the xylan and pectin fractions (described in Section 2.9) were quantified using the phenol-sulphuric acid assay (described in Section 2.10.1). Using a glucose standard curve the correct volume of KOH and ChASS fraction to yield 3 µg of sugar was dried in a speed-vac centrifuge. Once dried, 200 µl of 20% trifluoroacetic acid (TFA) was added to the sample. The samples were boiled for 2 hours at 121°C, and once cooled down were dried under nitrogen flow. Once the TFA was evaporated, the samples were washed twice with isopropanol, which was evaporated in the speed-vac each time.

Sugar samples from saccharification did not require hydrolysis. They were quantified using the phenol-sulphuric acid assay. As the objective of GC-MS was also to confirm the quantity of sugar present in the sample, uniform volumes equivalent to roughly 3 µg sugar were dried in a speed-vac. It was necessary that the samples to be analysed by GC-MS did not contain very much more than 3 µg of sugar as exceeding this number by any great amount would result in overloading the GC column.

2.14.2 N,O-bis-trimethylsilyl trifluoroacetamide (BSTFA) derivatization

Once the sample to be analysed by GC-MS was dried, 30 µl of pyridine containing 20 mg/ml of O-methylhydroxylamine hydrochloride and 10 µg/µl of internal standards was added and the mixture vortexed vigorously. The internal standards were tri-tert-butylbenzene, which remained inert for the whole analysis process, and 4-phenyl butrytic acid, which reacts with methylhydroxylamine hydrochloride and N,O-bis-trimethylsilyl trifluoroacetamide (BSTFA) to form a TMS-derivative. The samples were incubated at room temperature overnight to allow the methoximation of aldehyde and keto groups. Following the overnight incubation, the samples were transferred to glass vials and 30 µl of BSTFA+TMCS (N,O-bis-trimethylsilyl trifluoroacetamide, 1% trimethylchlorosilane) was added. The samples were then incubated at room temperature for 2 hours. The BSTFA reacted with the active hydrogen ions on the sugars in the sample, replacing them with trimethylsilyl groups. This reduces the number of stereoisomers in the sample. The chemical structures of glucose and glucose that has undergone methoximation and BSTFA derivatization are shown in Figure 17. After the incubation period was over, 30 µl of hexane was added to each sample.

2.14.3 GC-MS conditions

One microlitre of derivatized sample was injected in a splitless mode by the autosampler into the gas chromatograph. The data obtained were analysed with Chemstation software and mass spectra of the peaks were compared against known standards which were run alongside the unknown samples, as well as NIST 05. The solvent delay was set at 5 minutes. The injector and detector temperatures were set at 250°C and 280°C respectively. The oven temperature started at 50°C and rose to 320°C at a rate of 7.1°C/min. The mobile phase was helium, set at 10 psi.

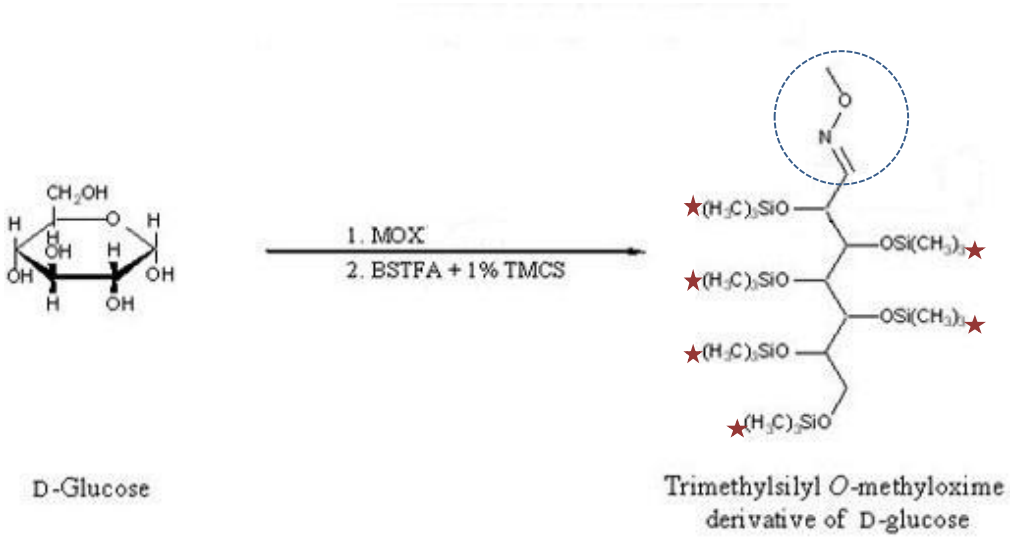


Figure 17 Methoximation and BSTFA derivatisation of glucose

Glucose undergoes a methoxymation reaction with O-methylhydroxylamine hydrochloride (MOX) which linearises the α and β forms of D-glucose, reducing the number of isomers in the sample. Methoximation replaces the aldehyde group on C1 with an $NOCH_3$ group (circled in blue). BSTFA reacts with H ions in –OH groups, replacing them with trimethylsilyl groups which are labelled with red stars.

Adapted from the University of Minnesota
http://ftp.gcrc.umn.edu/gcrc/services/cores/GC-MS.html accessed on 17 October 2011.

2.15 Statistical analysis

Throughout this project, a two-tailed type 2 Student's T-test was used to assess statistical significance between two sets of data gathered from independant samples which share a similar range. If the p value was lower than 0.05, the result was considered significant.

3 Profiling the effects of cell wall biosynthesis modification on transgenic tobacco lines

3.1 Fractionation and analysis of cell wall components

3.1.1 Transgenesis does not lead to a phenotypic change in levels of cell wall polysaccharides

It was important to investigate the effects of lignin and xylan down-regulation on the other cell wall components, both in the primary cell wall and in the secondary cell wall. As lignin and xylan are not present in primary cell walls, reducing their biosynthesis was not expected to have an effect on primary wall material extracted from leaves. Xyloglycan, the primary cell wall hemicellulose, makes up 20-25% of the primary cell wall but contains less xylose than its secondary cell wall counterpart glucuronoxylan, which makes up 20-30% of the secondary cell wall (Scheller et al., 2010). Changing lignin and hemicellulose content however has been demonstrated to have an effect on the levels of other secondary cell wall components (Lionetti et al., 2010; Brown et al., 2010; Lee et al., 2009; Persson et al., 2007; Chen and Dixon, 2007; Park et al., 2007; Jackson et al., 2003; Li et al., 2003; Hu et al., 1999). It was of extra interest if cellulose deposition was increased in any of the cell wall modified lines, as this increases the potential yield of fermentable sugar glucose from the biomass.

There were no changes in the composition of leaf tissue (Table 6), which was expected as the transgenesis was targeted to the secondary cell wall. Significant differences were observed in secondary cell wall composition. The acetyl bromide lignin content of lines *ccr, c4h* and *prx* was significantly different to that of the NVS wildtype. The reduction in lignin content in *ccr* and *c4h* from 21% in the NVS wildtype to 11% and 15% respectively was similar to previous analyses of these lines (O'Connell et al., 2002; Blee et al., 2001). The 9% lignin content in *prx*, a reduction of more than 50% compared to NVS, was a more extreme phenotype than the 15% lignin content reported by Blee et al. (2003).

Lignin content is notoriously difficult to determine. Klason lignin is the insoluble residue that is left over after pectins, hemicelluloses and cellulose have been removed from the cell wall material. This method often overestimates lignin content due to co-precipitation of proteins and the presence of non-lignin inorganic or phenolic

substances, which may remain even after sulphuric acid hydrolysis (Anterola and Lewis, 2002). The acetyl bromide lignin assay, in which the UV absorbance of lignin phenolics, solubilised by acetylation, is detected, also has a limitation. The extinction coefficient used to calculate mass from the UV absorbance may not be accurate for transgenic plants (Anterola and Lewis, 2002).

Klason quantification, which is labelled as insoluble residue in Table 6, resulted as expected in higher estimations of lignin content than acetyl bromide lignin analysis. This showed that though there were no significant differences in polysaccharide content between NVS, *ccr, c4h* and *prx*, there were different levels of non-lignin alkali- and acid-insoluble residue present in the cell wall. Both analyses showed that lignin content in *uxs* was increased and cellulose content was unchanged, confirming that of Bindschedler et al. (2007) stating that there is more lignin in *uxs* than in K326 while glucose levels were the same. The difference in acetyl bromide lignin content was significant according to the Student's T-test (p = 0.05). There were no significant differences between the polysaccharide contents of any of the transgenic lines and their wildtypes. This is consistent with both *CCR*-down regulated alfalfa lines, which had variable total carbohydrate content (Jackson et al., 2003); and with the *prx* analysis of Kavousi et al. (2010).

Table 6 Fractionation of wildtype and transgenic cell wall biomass. Data are the averages of three biological replicates, each with two technical replicates. The standard error is given. The data do not add up to 100 as there was not 100% recovery of starting biomass.

Line	% cell wall composition of leaf (mainly primary wall)			% cell wall composition of stem (mainly secondary wall)				% Acetyl bromide lignin
	Pectin	Hemi-cellulose	Cellulose	Pectin	Hemi-cellulose	Cellulose	Insoluble residue	
WT (NVS)	25 ± 2	17 ± 2	40 ± 1	< 4	22 ± 4	39 ± 2	25 ± 2	21 ± 3
c4h	26 ± 3	17 ± 2	40 ± 1	< 4	25 ± 2	44 ± 3	23 ± 2	15 ± 2
ccr	28 ± 2	15 ± 1	43 ± 3	< 4	22 ± 2	35 ± 3	21 ± 2	11 ± 3
prx	28 ± 3	16 ± 1	41 ± 2	< 4	24 ± 1	41 ± 2	18 ± 1	9 ± 2
WT (K326)	25 ± 2	17 ± 1	39 ± 2	< 4	20 ± 2	34 ± 1	21 ± 1	15 ± 2
uxs	26 ± 3	15 ± 3	42 ± 3	< 4	10 ± 1	32 ± 2	30 ± 3	18 ± 2

3.1.2 Lignin down-regulation affects monosaccharide composition of the cell wall

Composition of the cell wall polysaccharides is also of interest when studying the effects of cell wall modification, as the quantity of different monosaccharides can affect the cross-linking in the cell wall (Iiyama et al., 1994; Grabber, 2005; Mortimer et al., 2010). Additionally, a compensation mechanism may be observed in line *uxs* in which xylose biosynthesis is disrupted, where another monosaccharide replaces xylose in hemicelluloses and pectins. Such a mechanism has not previously been observed.

Xylose made up between 70 and 80% of the hemicellulose fraction. Glucuronic acid, glucose and mannose accounted for the remaining sugars (Figure 18A). This is consistent with the fact that glucuronoxylan, made up of a xylose backbone and glucuronic acid side-chains, is the most common hemicellulose in the dicot secondary cell wall (Ebringerova and Heinze, 1999). Glucose and mannose are the monosaccharides that make up glucomannan, which makes up 10% of secondary cell wall hemicelluloses (Scheller and Ulskov, 2010). The breakdown of monomers in hemicellulose, shown in Figure 18A, was similar to that seen in both Arabidopsis (Persson et al., 2007) and poplar (Nishikubo et al., 2007; Lee et al. 2007), although Akpinar et al. (2007) found the hemicellulose extract from tobacco stalk to be 93% xylose, uronic acid and glucose were also present.

Interestingly the *TOBACCO PEROXIDASE 60* down-regulated line *prx* had only 63% xylose in the hemicellulose extract, uronic acid apparently replacing it (Figure 18A). Lignin down-regulation is known to affect monosaccharide composition, as a similar effect was seen in *CCR* down-regulated poplar in which the xylose content increased (Chabannes et al., 2001) and in *CCR* down-regulated Arabidopsis, which showed differences in the levels of all non-cellulosic monosaccharides.

The sugar composition of the hemicellulose fraction did not vary between line *uxs* and the wildtype K326, despite the 50% reduction in its mass (Table 6). There was no compensatory proportional decrease in xylose, or increase in other hemicellulosic sugars such as mannose. This differs from *irx-15* Arabidopsis insertion lines in which xylan synthesis was disrupted. In these lines, xylose in non-cellulosic polysaccharides was reduced, and galacturonic acid content was increased (Brown et al., 2011). The reduction in xylose content was clearly a result of the antisense insertion however, as xylose was reduced to a greater extent than glucuronic acid, glucose or mannose.

The chelating agent-soluble (ChASS) extract, which contained mainly pectin, consisted of ~40% galacturonic acid (Figure 18). That uronic acids made up the largest percentage of the fraction was expected, as the α-1,4-linked galacturonic acid polymer homogalacturonan accounts for 65% of pectin. The presence of xylose, rhamnose, arabinose and galactose residues reflected the other major pectins in the plant cell wall: rhamnogalacturonan I, which has a galacturonic acid and rhamnose backbone and on average makes up 20-35% of pectins; rhamnogalacturonan II, on average 10% of pectin in cell walls; and xylogalacturonan. All these pectic polysaccharides have side chains consisting of rhamnose, arabinose and galactose residues. The sugars present and their percentages in NVS, *ccr, c4h, prx* and *uxs* compared well with published analyses of Arabidopsis stems (Brown et al., 2011), tomato (Spadoni et al., 2006) and tree species *Cryptomeria japonica* (Edashige and Ishii, 1996).

The *CINNAMATE-4-HYDROXYLASE* down-regulated line *c4h* contained 17% arabinose in the pectin fraction, compared to less than 11% in the wildtype NVS. The cell walls from lignin down-regulated lines *ccr, c4h* and *prx* contained less pectin than NVS while *uxs* had higher lignin content than the wildtype K326 (Figure 18). A significant reduction of pectic sugars in lignin down-regulated tobacco lines has previously been described (Abdulrazzak et al., 2006). Uronic acid and arabinose content of cell walls was reduced in tobacco plants with antisense suppression of *C3H*.

The cultivar K326 contained a higher proportion of xylogalacturonan than NVS. The xylose biosynthesis interruption in line *uxs* caused a reduction in xylogalacturonan as well as in xylan, which in turn was compensated for by increased deposition of other pectins such as homogalacturonan.

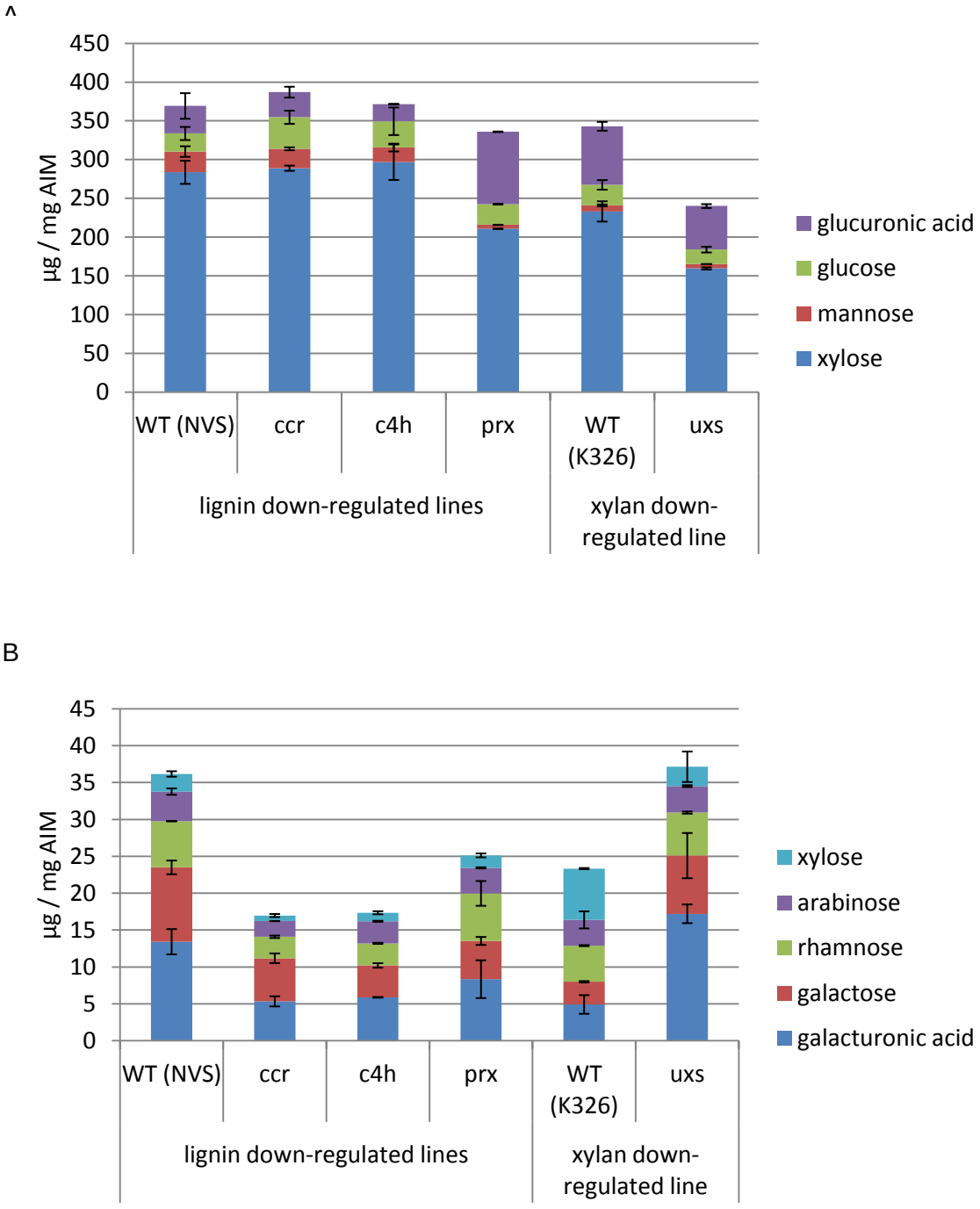


Figure 18 Monosaccharide composition of (A) hemicellulose and (B) pectin fractions extracted from secondary cell wall of WT and transgenic lines. Data given are the average of three biological replicates, each with two technical replicates. Error bars represent standard error.

A Xylose is the major hemicellulosic sugar. Mannose, glucose and glucuronic acid comprise the remaining sugars, reflecting the presence of glucuronoxylan and glucomannan in the cell wall. *prx* contains more glucuronic acid than the other lines.

B Galacturonic acid is the major pectic sugar; which is consistent with homogalacturonan being the principal pectin. Xylose, arabinose, rhamnose and galactose are the monosaccharides in xylogalacturonan, rhamnogalacturonan I and ramnogalacturonan II.

3.2 Modification of the lignin biosynthesis pathway leads to altered vascular structure

As secondary cell walls are necessary for providing strength to the vascular tissue of a plant, it was possible that the reduction in lignin or xylan content resulted in an altered vascular phenotype. This has been observed previously in plants in which cellulose (Taylor et al., 2003; Taylor et al., 1999; Turner and Somerville, 1997) or hemicelluloses had been down-regulated (Nishikubo et al., 2011; Peňa et al., 2007).

The *TOBACCO PEROXIDASE 60* down-regulated line *prx* was the only line that has an abnormal phenotype when observable without magnification (Figure 19). The stem cross-section of *prx* had a thinner purple stain than NVS, indicating that this line had a much thinner lignified xylem ring than wildtype. The *prx* line has previously been described as having 28% reduction in xylem development on a dry weight basis (Kavousi et al., 2010). The reduction of lignin in the vascular bundle may account for the increased diameter of the stem. When viewed at x20 magnification under a microscope, there were differences between NVS and *ccr* and *c4h,* as well as *prx* (Figure 19). While the vessel elements in NVS were uniform in size and evenly distributed, their sizes in the lignin down-regulated lines were very variable. In all three, there is an uneven distribution of vessel elements, with lines of two or more large vessel cells frequently forming along a ray. It is possible that the reduction in lignin deposition allows the cells to expand beyond the normal expansion period, so parenchyma cells have a variable size.

There was no obvious difference between the xylem structure of xylan down-regulated line *uxs* and the K326 wildtype (Figure 19). This confirms the analysis of Bindschedler et al. (2007).

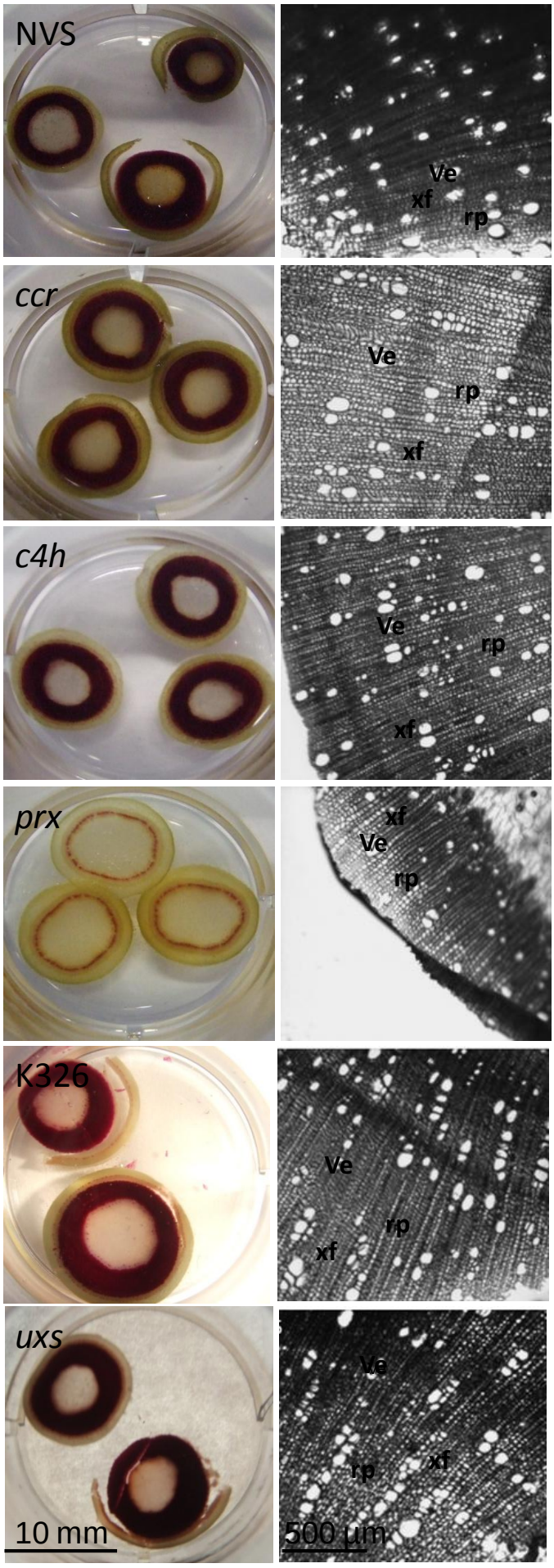


Figure 19 Stem sections from internode 3 of lignin-modified tobacco lines stained with phloroglucinol.

NVS (wildtype) and lines down-regulated in CCR (*ccr*), C4H (*c4h*), TP60 (*prx*); and wildtype K326 and UXS down-regulated *uxs*.

prx had a thinner purple stain than NVS, indicating that this line had a much thinner lignified xylem ring than wildtype.

Images are representative of three biological replicates.

Ve Vessels; xf xylary fibre; rp ray parenchyma.

3.3 Cell wall modifications change the saccharification properties of biomass

3.3.1 Overview of methods and rationale

Saccharification assays measure the quantity of sugars released from biomass over a given amount of time. In this case specifically, soluble sugars released from acetone insoluble cell wall material (AIM) by cellulase enzymes will be quantified. The differences between the transgenic lines will confirm whether or not lignin and xylan modification have an impact on sugar release, and consequentially on biofuel production.

There are three types of cellulases: exoglucanases which cleave two to four glucose units from the ends of chains; endoglucanases which break internal bonds; and cellobiases which hydrolyse the small chains of monomers into single glucose molecules. All three types were included in the saccharification assays described here. The enzyme cocktail also included a crude cell wall degrading mixture containing hemicellulases and pectinases.

The phenol sulphuric acid assay was used to quantify total carbohydrates in a sample (Fry, 1988). Sulphuric acid hydrolyses the glycosidic bonds between sugars, producing furfural or a furfural derivative. These molecules undergo a condensation reaction with phenol to form coloured compounds. The amount of sugars present is proportional to the absorbence of the final coloured solution (Ahmed, 2004).

Finally the sugars released by the enzymes were identified by gas chromatography-mass spectrometry (GC-MS), which separates the molecules according to mass and polarity in the gas chromatograph. The mass spectrometer then ionizes the molecules to give structural information with which the compounds can be identified.

3.4 Optimisation of Saccharification Assay

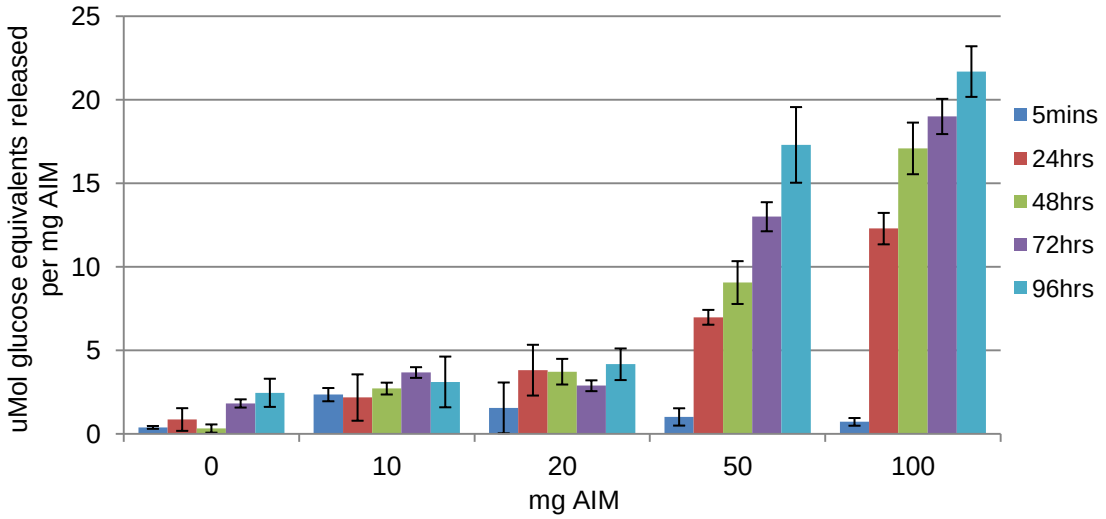
Initial attempts at saccharification were carried out in 10 ml glass tubes, as recommended by Selig et al. (2008). These experiments were contaminated by a yeast and consequentially the results appeared random. The experiments were scaled down to fit into 1.5ml Eppendorf tubes and 0.2% sodium azide (Selig et al., 2008) was replaced with the antibiotic cocktail mentioned in Section 2.5.2. No evidence of any

microorganism growth could be found. At this point the ratio of enzymes, AIM and buffer were as recommended in Selig et al. (2008). Sugar release between experiments was consistent.

Assays in which sugar release from 10, 20, 50, and 100 mg of NVS AIM by 8U of cellulases were carried out in order to assess the optimum mass of AIM for this experiment. The maximum amount of AIM was 100 mg as the total assay volume could not exceed 1 ml. Early trials showed that Eppendorf tubes, not larger glass tubes, are ideal as they reduce the risk of microbial contamination and are easier to agitate at an elevated temperature for a number of days.

As shown in Figure 20, 100 mg of AIM released the highest concentration of soluble sugars at all points on the timecourse. Both 50mg and 100mg of AIM released sugars at a fairly constant rate over 96 hours. Soluble sugars in the enzyme control tubes, which were used to blank the reaction tubes, meant that the concentration of soluble sugars released from 10 mg and 20 mg of AIM appeared random over the time course. The sugar released from AIM alone was not enough in these samples to overcome the background reading. This background reading occurred because Driselase, a cell wall digesting enzyme preparation from Basidiomycete sp., includes a selection of cell wall degrading enzymes, including proteases. The proteases in Driselase digested the cellulases, some of which are glycosylated. The glucan groups may be detected by the phenol sulphuric acid test. Throughout the remainder of this chapter, the enzyme background reading is not presented on the graphs but was deducted from the readings before the data was converted into µMol/mg AIM.

A



B

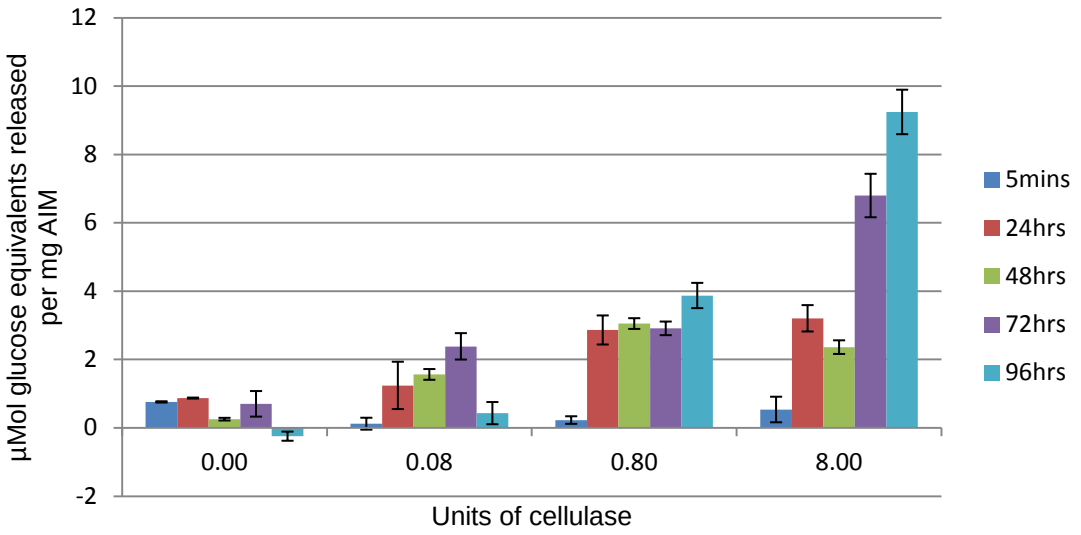


Figure 20 Optimisation of saccharification assays.

A Optimisation of mass of AIM in the assay. 10, 20, 50 or 100 mg of AIM was hydrolysed by 8U cellulase in 1ml total assay volume. Sugar release was quantified every 24 hours. 50mg AIM is the ideal mass in the saccharification assays.

B Optimisation of units of enzyme in the assay. 8U 0, 0.08, 0.8 or 8.0 units of cellulase were added to 50 mg AIM in a 1ml saccharification assay volume. Sugar release was quantified every 24 hours.

Data given are from two biological replicates, each with three technical replicates. Error bars represent standard error.

Units of cellulase were also optimised. One unit is defined by Sigma-Aldrich as enough cellulase to liberate 1.0 µMol of glucose from cellulose in one hour at pH 5.0 at 37°C with a 2 hour total incubation time. The NREL protocol (Selig et al., 2008) advises an enzyme concentration of 60 U per gram of cellulose in a total assay volume of 10 ml. This equates to 3 U for 50 mg AIM. In order to confirm whether this figure would be suitable when the protocol was adapted for 50 mg AIM with total assay volume 1 ml, enzyme cocktails that containing no enzymes, 0.08 U, 0.8 U and 8 U units were added to 50mg of tissue. The concentration of soluble sugars was tested at 24 hour intervals. The results are shown in Figure 20.

Again the concentration of sugars in solution increased at a constant rate, indicating that the enzymes were working at optimum conditions for at least 96 hours. There was no significant change in sugar content of the supernatant in the control sample in which AIM was incubated with no enzyme. The samples incubated with 0.08U of enzyme did not show consistent release of sugar. The sugar in the supernatant appeared to decrease from 2.2 µg/mg AIM to 0.2 µg/mg AIM in the final 24 hours, which is impossible as the system was sterile and no more liquid was removed from the tube at this time point than at any other. This reduction in sugar quantity suggests the change in sugars released by 0.08 U enzyme over 96 hours was below the limit of detectability. The increase in sugar release over time by 0.8 U of enzyme was not constant. However 8 U enzyme released sugars consistently over 96 hours.

The final reaction components of the saccharification assay are detailed in Section 2.10. Although more sugar was released from 100mg of AIM than from 50mg, it was crucial that the experiments could be carried out in Eppendorf tubes which meant that 100mg was not suitable. The units of cellulase used was 8U as at this concentration the sugars released from the AIM were enough to be observed over the background sugar release from the enzyme control tubes.

Figure 20 shows that the increase in sugar concentration from T0 to 96 hours was constant. The enzymes were still active and had not reached saturation. For ease and speed of planning the 72 hours time point was chosen as the standard duration of the saccharification assays.

3.5 Sugar release from leaf tissue is unaffected by secondary cell wall modification

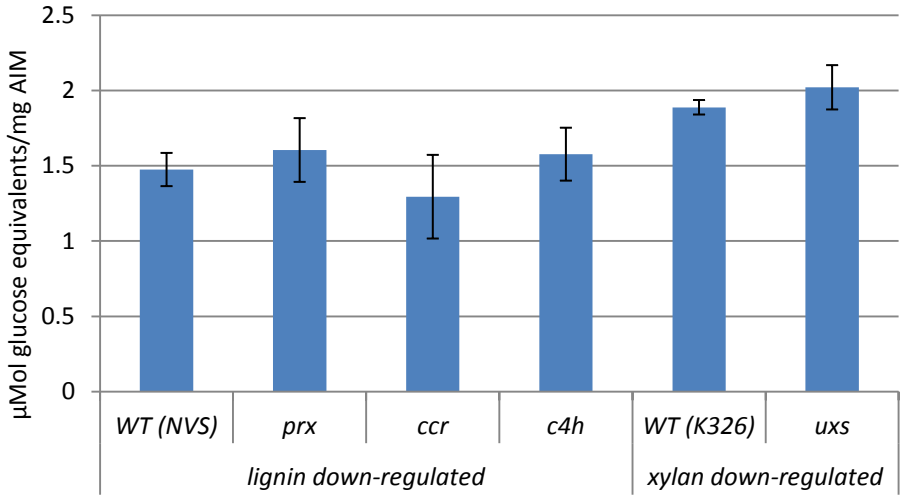


Figure 21 Sugar release from leaf tissue over 72 hours does not change significantly between tobacco lines. For each sample, 50mg of AIM was treated with 40U cellulase. There was no significant difference in sugar release. Data given are the average of three biological replicates, each with three technical replicates. Error bars represent standard error.

There was no significant difference in quantity of sugars released by saccharification of leaf cell wall material from different tobacco lines (Figure 21). The transgenic lines had altered lignin and xylan content, indicating that secondary cell walls such and not primary cell walls, would be affected (Table 6). Secondary cell walls are present in a limited number of cell types such as tracheary elements (Zhong and Ye, 2009b) which form a major part of 'woody' mature tobacco stem but do not occur as frequently in leaf tissue. As a result any difference in sugar release from cell walls was expected to be from secondary cell walls, in which lignin and xylan down-regulation would increase exposure of cellulose to the enzymes. Modification of primary cell wall structure by pectin alteration results in improved saccharification of leaf tissue (Lionetti et al., 2010).

3.6 Lines *prx* and *uxs* show improved sugar release from secondary cell wall material

The objective of the saccharification assays was to ascertain the effect of secondary cell wall modification on the ease of soluble sugar release from cell wall polysaccharides. Having established that there was no significant difference in sugar release per mg of acetone insoluble cell wall material (AIM) from primary cell wall enriched leaf tissue, secondary cell wall from tobacco stem was extracted and subjected to saccharification.

As seen in Figure 22, 30% more soluble sugars were released from *TP60* down-regulated *prx* AIM than from NVS. Line *prx* had 23% reduction in lignin as compared to NVS (Blee et al., 2003); the same reduction as *ccr* (O'Connell et al., 2002) and a slightly smaller reduction in lignin content than 27% in *c4h* (Blee et al., 2001) which showed no improvement in saccharification. However, cell wall fractionation and acetyl bromide lignin content analysis of the AIM used here indicated that *prx* had significantly lower lignin content than the other lines (Table 6).

The xylan down-regulated line *uxs* also showed a significant (p=0.028 according to Students T-test) improvement in saccharification, demonstrating that hemicellulose modification is a feasible means of improving sugar release from biomass.

When considering saccharification efficiency (Table 7), that is the percentage of total carbohydrates released by the enzyme, the difference between lines *prx* and *uxs* and the wildtypes became more pronounced, increasing to a twofold improvement, due to the reduction in total carbohydrate in these lines. 58.4% of cell wall carbohydrates were released from *prx* compared to 32.6% in NVS, while the other wildtype K326 showed 13.6% saccharification efficiency and from line *uxs*, 25.1% of polysaccharides were hydrolysed.

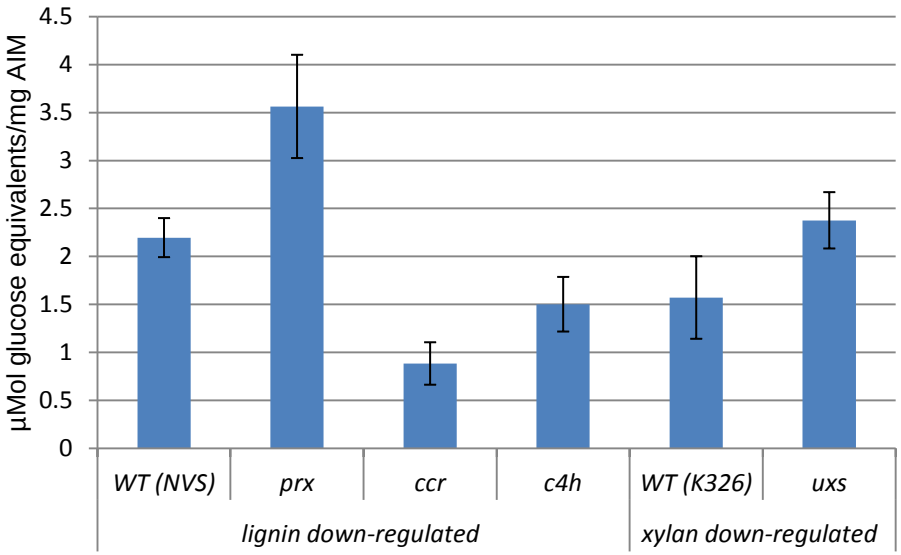


Figure 22 Sugar released from 50 mg stem AIM after 72 hours saccharification with 40U cellulase. There are significant differences between the lignin and xylan down-regulated lines and their wildtypes. Data given are representative of eight biological replicates, each with three or four technical replicates. Error bars represent standard error.

Table 7 Sugar release from AIM as a percentage of total carbohydrate in 1 mg of AIM. Data given are representative of eight biological replicates, each with three or four technical replicates. Error bars represent standard error.

Line	µMol sugar released per mg AIM	µMol total carbohydrate per mg AIM	% of total carbohydrate released per mg AIM
NVS	2.19	6.73	32.6
prx	3.56	6.10	58.4
ccr	0.88	4.46	19.8
c4h	1.50	7.78	19.3
K326	1.57	11.56	13.6
uxs	2.38	9.46	25.1

3.7 Glucose is the main monosaccharide released by enzymatic saccharification

Throughout the saccharification timecourse, the monosaccharide representing the largest proportion of sugars released was glucose at about 80% (B

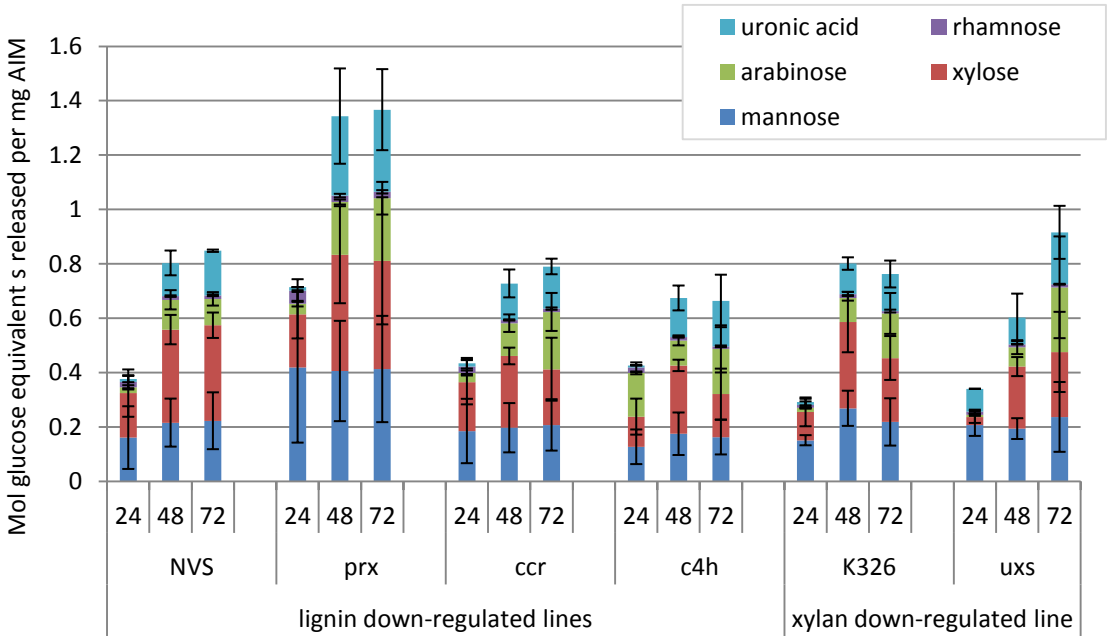


Figure 23), showing that cellulose was the main polysaccharide hydrolysed. This reflects the enzyme preparation used to digest the cell wall material, which was predominantly cellulase, as well as the fact that cellulose is the main cell wall carbohydrate. Cellulose accounted for between 30-45% of the cell wall in all the tobacco lines analysed (Table 6).

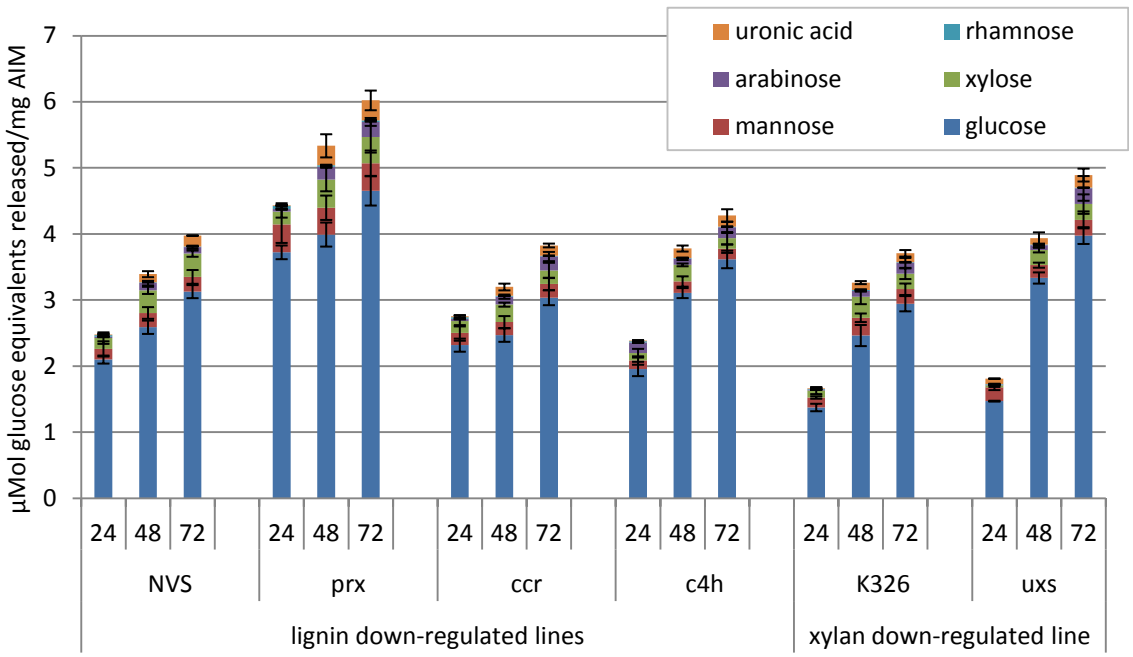
The remaining 20% of sugars were xylose, mannose, arabinose and uronic acids. These are all monomers of both pectins and xylans. The dominance of xylose and mannose at 24 hours suggests that hemicelluloses are the first non-cellulosic polysaccharides to be hydrolysed by the cell wall degrading enzymes, as secondary cell wall hemicelluloses are primarily xylans and mannans (Ebringerová, 1999).

Interestingly, glucose content dropped by 6-8% in NVS, *ccr, prx*, and K326 after 48 hrs. Non-cellulosic sugars uronic acid, arabinose, xylose and mannose accounted for a larger proportion of the sugars released than they did in 24 hours. This suggests that as cellulose is hydrolysed, hemicellulose and pectin become exposed. By 72 hours glucose had regained the 80% majority; the xylans and pectins were all hydrolysed while cellulose was still being degraded.

CINNAMATE-4-HYDROXYLASE down-regulated line *c4h* was the only lignin-modified line with a different digestion profile to the wildtype. Arabinose made up 6% of the sugars released at 24h, compared to less than 1% in the other lines. Arabinose also made up a greater percentage of the ChASS fraction in *c4h* than in the other lines (Figure 18B), suggesting that the suppression of *C4H* caused an increase in arabinose residues in secondary cell wall pectin.

The xylan down-regulated line *uxs* also differed from the wildtype, line K326. There was 1.7% xylose in the sugars released after 24 hrs, compared to 6.3% in K326. This was due to the reduction in hemicellulose in this line (Table 6); the xylose from hemicellulose was absent while the xylose residues in pectin have been released. By 48 hrs however, the limited xylan is exposed and the xylose:glucose ratios are equal in *uxs* and K326.

A



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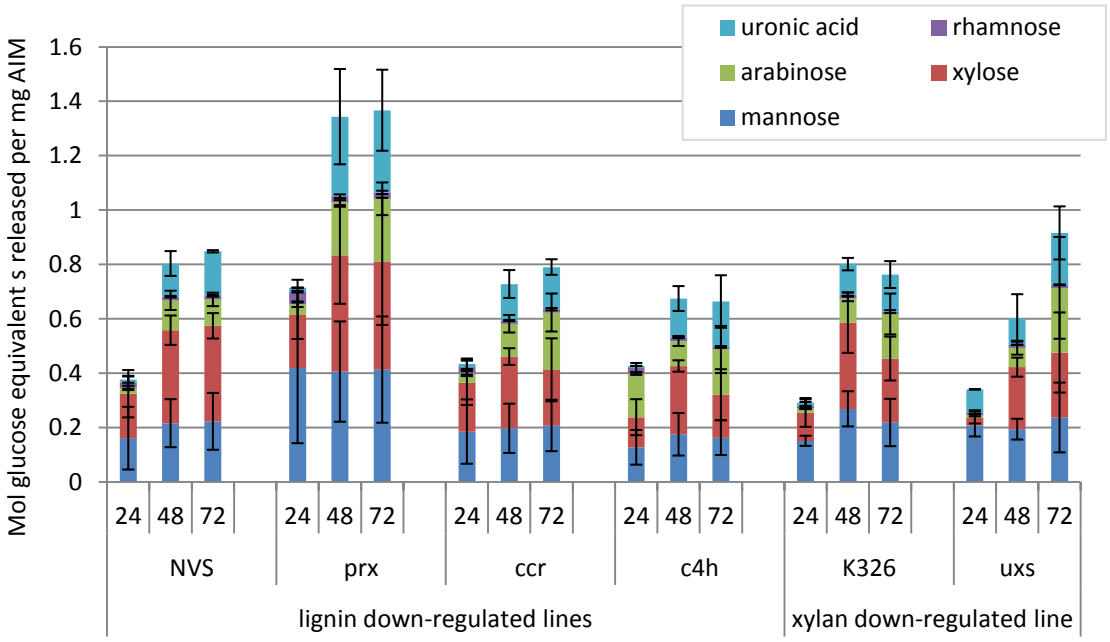


Figure 23 Sugar composition of sugars released from cellulolytic saccharification over a time course of 72 hours.

A All monosaccharides,; B monosaccharides without glucose Data given are the average of two biological replicates, each with two technical replicates. Error bars represent standard error.

3.8 Discussion

3.8.1 The effect of lignin and hemicellulose down-regulation on the plant phenotype

Cell wall modifications are known to affect xylem structure, so the differences observed in the xylem tissue of lines *ccr, prx* and *c4h* (Figure 19) are to be expected given the extent of lignin down-regulation in these lines, shown in Table 6. Transgenic Arabidopsis lines deficient in cellulose synthesis have irregular xylem (*irx*) phenotypes (Turner and Somerville, 1997; Taylor et al., 1999; and Taylor et al., 2003). Similarly hemicellulose down-regulation causes irregular xylem formation (Peňa et al., 2007). Though in the case of irregular xylem mutants the irregularity is in the form of collapsed xylem vessels, it is also documented that cell wall modification can cause increased vessel element size; xyloglucan rearrangements cause larger vessel elements in aspen (Nishikubo et al., 2011). While the irregular xylem phenotype can cause dwarfism (Taylor et al., 2003; Brown et al., 2005; Brown et al., 2007, Peňa et al., 2007), it is clear from the results presented here that it is possible for lignin and xylan down-regulation to have no effect on the healthy phenotype of a plant.

In *prx* the xylem tissue was reduced (Figure 19; Kavousi et al., 2010), but this change has no ill effect when the plants are grown in a controlled environment. It is already established that changes to the vascular structure do not need to result in major physical abnormality. Arabidopsis *irx* mutants with reduced cellulose content had phenotypes ranging from slightly shorter stems to no differences from the wildtype (Turner and Somerville, 1997). Nishikubo et al. (2011) described aspen trees which had large vessel elements due to reduced hemicellulose content, and were slightly shorter than the wildtype but otherwise healthy. Indeed in line *prx* there were some abnormalities in the leaves but no characteristics were observed that would affect the health of the plant (Kavousi et al., 2010). Stomatal conductance was unaffected by transgenesis (Kavousi et al., 2010). Similarly all the cell wall modified lines presented here have healthy phenotypes when grown in the glass house.

3.8.2 Lignin and hemicellulose down-regulation have opposite effects on the cellulose content of secondary cell walls

There is an on-going debate over whether cell wall biosynthesis regulation can compensate for the suppression of one cell wall component by the over-deposition of another. Of particular interest for biofuel production is the question of whether lignin, hemicellulose or pectin down-regulation causes increased cellulose deposition in

response. The results presented here in Table 6 provide answers to these questions. While lignin content was reduced in *ccr, c4h* and *prx*, there was no difference in the polysaccharide content in these lines. Conversely the *uxs* line*,* in which *UDP-GLUCURONATE DECARBOXYLASE* down-regulation caused xylan deposition to halve, showed lignin content increasing from 21% in the K326 wildtype to 30% in *uxs*.

An overview of current literature presents a similar story. There are published results that suggest there is a compensation mechanism to replace lower than normal cell wall components in modified lines, and others that show no change in levels of non-target cell wall molecules. *CCR*-down regulated alfalfa lines had variable total carbohydrate content (Jackson et al., 2003), while reducing pectin content has no effect on total sugar content in the primary cell walls of Arabidopsis (Lionetti et al., 2010). Suppression of xylan synthesis in Arabidopsis actually reduced cellulose content in a manner apparently proportional to xylan content (Brown et al., 2010). Reduction of glucuronoxylan content caused slightly lower glucose levels in the cell walls of mature Arabidopsis stems (Persson et al., 2007) and poplar lines suppressed in xylan biosynthesis had reduced total sugar content in the cell wall (Lee et al., 2009). Hu et al. (1999) found that cellulose deposition increased in compensation for reduced lignin due to the suppression of *PAL* and *CCR* in poplar. Li et al. (2003) confirmed this in *4CL* and *CONIFERALDEHYDE 5-HYDROXYLASE* down-regulated aspen, in which cellulose content was increased. Similarly Chen and Dixon (2007) observed higher total carbohydrate levels in lignin down-regulated alfalfa lines. An increase in cellulose content seemingly in response to hemicellulose manipulation was also observed in the cell walls of tobacco expressing xyloglucanase from *Aspergillus niger* (Park et al., 2004). Although xyloglucan is a primary cell wall carbohydrate, the 10% increase in cellulose content was observed in the secondary cell wall in addition to the primary wall. In reverse, though still in support of the theory that reducing one cell wall component will cause increased deposition of another, it has been shown that decreasing cellulose synthesis by suppressing CESA3 expression leads to higher lignin deposition in Arabidopsis (Caño-Delgado et al., 2003).

With evidences on both sides of the argument, it seems that there is no automatic compensation mechanism for increasing cellulose content to replace lignin or non-cellulosic cell wall polysaccharides. When considering the lignin and cellulose content of hemicellulose modified plant lines, the pathway position of the target of down-regulation appears to influence on cell wall composition. UXS synthesises the direct precursor to xylan, while proteins encoded by *IRX8* and GT47, the genes down-regulated by Persson et al. (2007) and Lee et al. (2009) respectively, have as yet

undefined roles in xylan biosynthesis. Conversely, tobacco plants in which xyloglucanase expression reduced hemicellulose content by degrading xyloglucan that had already been deposited showed increased cellulose content (Park et al., 2004). Possibly the elimination or reduction of xyloglucan in the cell wall matrix triggered a response from cellulose synthesis which did not occur in plants whose cell walls had always contained less hemicellulose. In *uxs,* the compensation for xylan reduction is increased lignin deposition; perhaps lignin and hemicellulose synthesis are more closely co-ordinated with each other than with cellulose. This would reflect cross-links in the cell wall, which are more frequent between non-cellulosic polysaccharides and lignin than between cellulose and the other cell wall components. Lignin can link to cellulose only through ether bonds between hydroxyl groups, but the variety of monosaccharides present in hemicellulose molecules enables lignin to bond to hemicellulose via ether, ester and acetal bridges (Achythan et al., 2010). It is also worth noting that interrupting xylose biogenesis by suppressing *UXS* causes an irreversible reduction of xylan content which is not compensated for by arabinogalactan, mannan or another hemicellulose.

It is important to consider that the plant may compensate for reduced cell wall strength caused by cell wall modification by changing the structure of the components in the cell wall matrix. Cross-links from hemicellulose to cellulose and lignin are vital for cell wall integrity (Iiyama et al., 1994; Grabber, 2005; Mortimer et al., 2010). Grabber (2005) suggested that plants may increase the amount of cross-linking in the cell wall matrix in response to reduced lignin content. This is demonstrated here by the difference in saccharification properties between lignin down-regulated lines *ccr, c4h* and *prx*. Sugar release from *prx* is significantly higher than from the wildtype, while it is low in *ccr* and *c4h* (Figure 22). As all three lines have reduced lignin content (Table 6; Blee et al., 2001; O'Connell et al., 2002; Blee et al., 2003), lignin cannot be vital for cell wall resistance to hydrolysis. There may be increased cross-linking in the cell wall which increases recalcitrance. The difference in monosaccharide composition in the cell wall fractions of the lignin modified lines, seen in Figure 18, suggests changes in cell wall composition that is not reflected in the masses of hemicelluloses and pectin extracted, but may explain the resistance to enzymatic saccharification.

3.8.3 The effects of lignin and hemicellulose down-regulation on cell wall polysaccharide composition

Xylose in the *prx* hemicellulose fraction was low compared to NVS, *ccr* and *c4h*. The xylose was apparently replaced by uronic acid residues (Figure 18). This may be a response which increases cross-linking from xylan to cellulose and lignin. Uronic acids have been suggested as one half of a covalent ester bond to hydroxyl groups in lignin (Iiyama et al., 1994), and glucuronoxylan lacking in glucuronic acid residues is more amenable to extraction and hydrolysis than wildtype glucuronoxylan (Mortimer et al., 2010). However the increase in uronic acid residues in hemicelluloses was not sufficient to compensate for the reduction in lignin content; saccharification was more effective in the *prx* line than in wildtype.

Down-regulation of xylan by suppressing expression of glycosyl transferase genes led to decreased deposition of pectin in Arabidopsis (Mortimer et al., 2010; Persson et al., 2009). Line *uxs* did not show reduced levels of pectin or different pectin composition compared to the K326 wildtype. However, the xylan down-regulated line in the research presented here is suppressed in the xylose synthase gene *UXS*. While glycosyl transferases are involved in both xylan and pectin synthesis (Persson et al., 2007), limiting xylose synthesis does not necessarily mean a reduction in pectin, as xylogalacturonan can be replaced with homogalacturonan and xylose is not the major pectic monosaccharide. The increase in pectin content in *uxs* confirmed the existence of the cell wall compensation mechanism, as pectin is increased in response to the reduction of xylan content.

Similarly, pectin from *c4h* had more arabinose than the other pectin fractions (Figure 18). Pectin and lignin perform the same function; they both act like ‘glue’ in the primary and secondary cell wall matrix respectively. If lignin deposition is reduced, it is possible that pectin changes in response, possibly increasing a particular monosaccharide, as in this case, in order to enable more cross-linking. Arabinose-containing rhamnogalacturonan II cross-links with homogalacturonan, and this is important for the formation of the pectic macromolecular network and the structural integrity of the cell wall (Mohnen, 2008). Similarly homogalacturonan I was found to be up-regulated in cellulose deficient Arabidopsis plants, and the authors suggested the decrease in highly branched rhamnogalacturonan I and increase in homogalacturonan, which forms strong cross-links with itself, was a reactive measure to improve cell wall integrity when cellulose was absent (His et al., 2001).

It seems clear that changing lignin composition can improve saccharification. The syringyl/guaiacyl (S/G) ratios of lignin in *ccr* and *prx* are 1.71 and 1.21 respectively; higher than NVS at 0.82 (O'Connell et al., 2002; Blee et al., 2003; Kavousi et al., 2010). S/G ratios vary between plant species and tissue type (Anterola and Lewis, 2002). In general, lignin with high S/G ratio has improved pulping characteristics (Piquemal et al., 1998). In C3H down-regulated alfalfa plants, P (*p*-hydroxyphenyl) units are a substantial proportion of lignin composition at 55%, while wildtype alfalfa lignin contains 2.5% P units. As a result the cell wall digestibility is improved; 23.6% of wildtype cell wall remains after enzyme digestion while only 14.7% is left over from C3H suppressed cell walls (Ralph et al., 2006).

In this study, the *prx* line was the lignin down-regulated line with the greatest reduction in lignin content (Table 6). The *prx* line also had the most different xylem phenotype (Figure 19) and significant improvement in saccharification (Figure 22). It is apparent that the increased arabinose in *c4h* pectin was more successful at preventing cellulose hydrolysis than the changes in cell wall composition in *prx*. It seemed that low lignin content resulting from the prevention of lignin polymerisation had a much greater effect on the plant than merely interrupting lignin biosynthesis within the phenylpropanoid pathway.

3.8.4 Preventing lignin polymerisation improves sugar release from cell walls

It is evident from the data presented here that simply reducing lignin content does not improve crops for biofuel production. Reducing lignin content by down-regulating *TP60*, a peroxidase involved in final polymerization of monolignols, increases sugar release by 30% (Figure 22), but suppression of *C4H* and *CCR* makes sugars more inaccessible.

This is the first study analysing the effects of preventing lignin polymerisation. Previous research has been done on reducing lignin content by down-regulating phenylpropanoid enzymes. In *CCR* and *CAD/CCR* down-regulated tobacco lines, 50% more sugar release was observed than in the wildtype but the *cad/comt* surpressed lines showed no difference in saccharification properties (Gomez et al., 2010). In two out of three *CCR* suppressed alfalfa lines, there was nearly twofold improvement in saccharification efficiency but one *ccr* and all *cad* lines showed no improvement as compared to the wildtype line (Jackson et al., 2008). Lignin content was negatively correlated with sugar release from undomesticated poplar ecotypes, but even here

there were exceptions where material from trees with average lignin content had high sugar release after enzymatic treatment (Studer et al., 2011).

This work demonstrated that lignin down-regulation by preventing polymerization of monolignols had an important effect on saccharification. The data presented here also reinforced published research showing that down-regulating other lignin synthesis genes may not improve cellulose accessibility, despite the lignin content being reduced.

The xylan down-regulated line *uxs* also showed improvement in saccharification though the increase in sugar release was not as large as in *prx*. This is consistent with other research, in which xylan down-regulated poplar line *gt47* showed up to 30% improvement in sugar release, while one of the lines showed no increase (Lee et al., 2009). Xylan down-regulation successfully improves the saccharification properties of a plant.

4 Pretreatment of stem cell wall material with *Phanerochaete chrysosporium*

4.1 Aims

As outlined in section1.5, a number of cell wall degrading microorganisms can be used to pretreat lignocellulosic material prior to saccharification and fermentation. *Phanerochaete chrysosporium* is a white rot fungus that metabolises lignin. The objective of this research was to ascertain what effect pretreatment with *P. chrysosporium* has on the saccharification efficiency of the cell wall modified tobacco lines.

4.2 *Phanerochaete chrysosporium* strain 24725 can grow on cell wall material

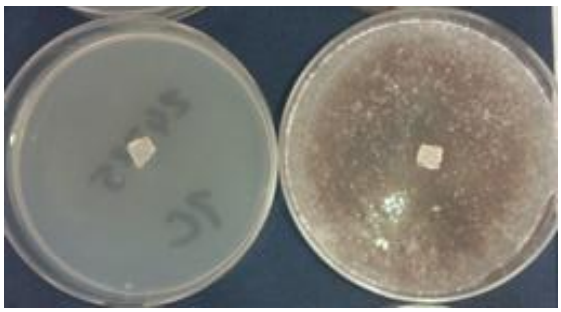


Figure 24 Growth of *Phanerochaete chrysosporium* strain 24725 on solid medium with cell wall material as the only nutrient source. The control medium containing agar with no nutrient source is shown above.

In order to confirm that *Phanerochaete chrysosporium* strain 24725 can metabolise cell wall material, the fungus was grown on an agar plate containing acetone insoluble cell wall material (AIM). The fungus was able to grow with AIM as the sole carbon and nutrient source, while on a plate comprising agar alone there was no growth (Figure 24). Having established that *P. chrysosporium* 24725 is able to metabolise cell walls, a fungal pretreatment protocol was developed.

4.3 Selection of optimum pretreatment method

Initial pretreatment trials were carried out on the K326 wildtype cultivar according to a method adapted from Keller et al. (2003). Acetone insoluble cell wall material (AIM) pretreated with this method did not show improved saccharification characteristics (Figure 25).

Pretreating K326 AIM using a method described by Tien and Kirk (1988) yielded more promising results (Figure 26). While both protocols use a culture medium containing glucose, KH_2PO_4, $MgSO_4$ and $FeSO_4$, the second protocol also included a trace element solution in the culture medium that contained $MnSO_4$, NaCl and $CuSO_4$, thiamine and also 0.4mM veratryl alcohol.

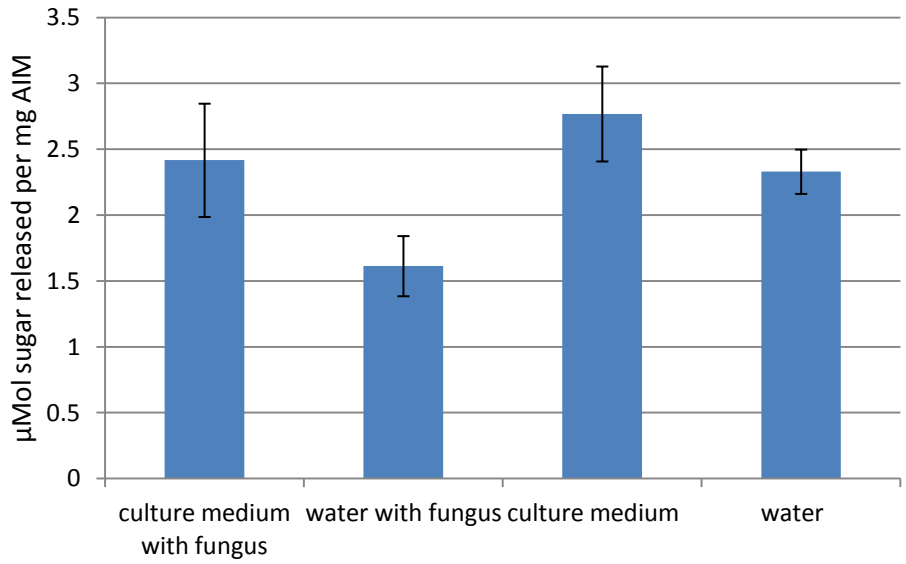
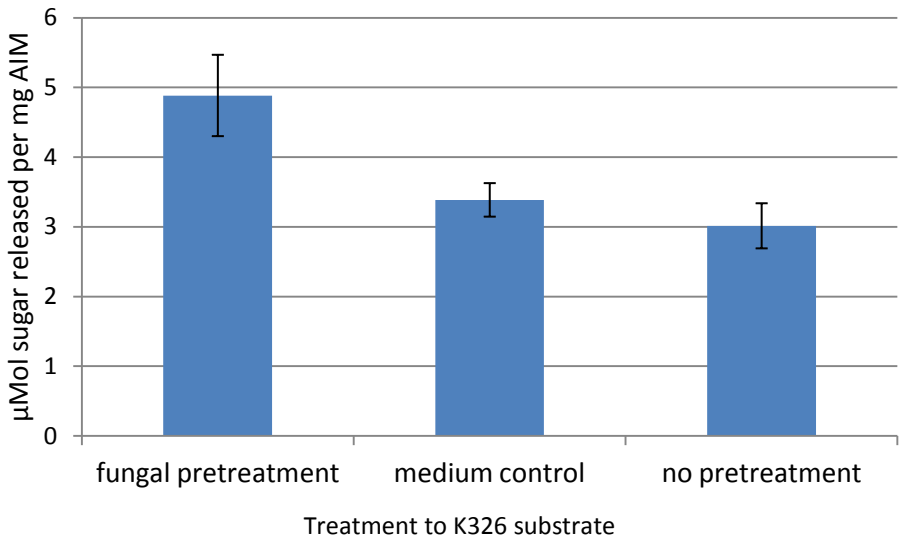


Figure 25 There was no significant difference between the sugar released from pretreated cell wall material and the control samples. AIM was incubated for 10 days with inoculated culture media, incolulated water, and medium and water controls. After three days cellulolytic saccharification there was no difference in sugar release between treatments. Data given are representative of five biological replicates, each with three or four technical replicates. Error bars represent standard deviation.

The results of two pretreatment methods using the same culture medium but under stationary or shaking conditions are shown in Figure 26. Pretreatment of acetone insoluble cell wall material (AIM) with *Phanerochaete chrysosporium* under both

shaking and static conditions resulted in improved saccharification of *Nicotiana tabacum* cultivar K326 AIM.

A



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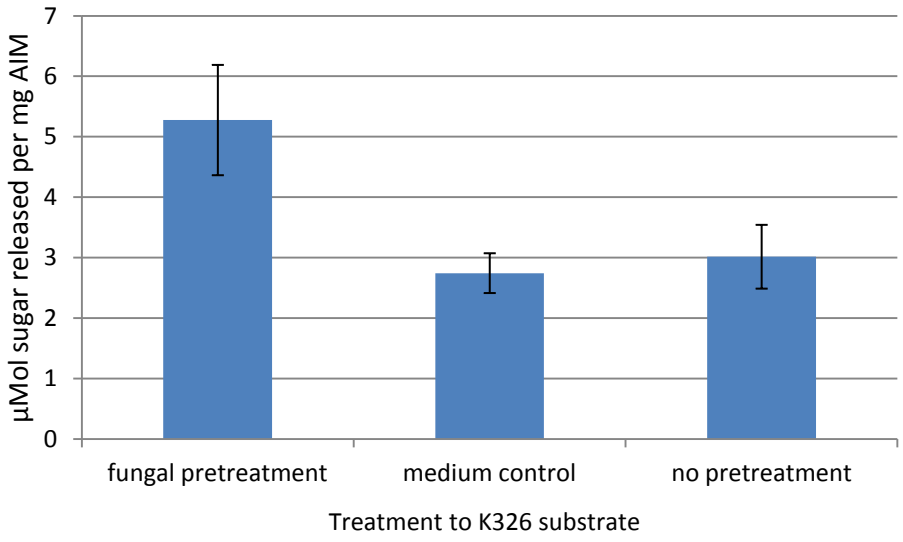

Figure 26 Saccharification of AIM pretreated with the stationary or pretreatment method is more effective than on control samples. Sugars released by cellulolytic saccharification of WT (K326) AIM after fungal pretreatment or incubation with control medium or water (A) on shaking incubator and (B) in stationary incubator. Data given are representative of two biological replicates, each with three technical replicates. Error bars represent standard deviation.

The optimum method was found to be the stationary protocol, as the culture medium without the fungus appears to have no effect on the AIM. Though not significant, the slight increase in sugar release from AIM homogenized in culture medium increased the variability of the results from the non-pretreated control samples. This may have been due to the constant agitation of the AIM within the medium which increased exposure to water, as well as salt ions and vitamins. Ten days under these conditions allowed the cell walls to swell under osmotic pressure, slightly improving saccharification. Additionally Tien and Kirk (1988) observed that agitated cultures had lower reproducibility and reliability.

A second reason for continuing with stationary cultures was that high cellulose degradation has been observed in cell wall material pretreated in agitated cultures. Shi et al. (2009) found that after 10 days treatment in a stationary culture, 21% of glucan was available, a drop of only 3% compared to un-pretreated material. After 10 days in the agitated culture, glucan availability was 11.7%.

In order to confirm that the growth of *Phanerochaete chrysosporium* was the same on different AIM substrates, RNA was extracted from the mycelial mat that formed on NVS, *prx,* K326 or *uxs* AIM after pretreatment. The gene expression of a control gene, 18S RNA, and a α-tubulin, the expression of which indicates mycelial growth (Tarkka et al., 2006) were compared. As seen in Figure 27, the expression of both the control gene 18S RNA and the mycelial growth indicator α-tubulin were the same in *Phanerochaete chrysosporium* grown on NVS, *prx,* K326 and *uxs*. These results confirmed that after the 10 day incubation period, the fungus had grown to the same extent on the different substrates.

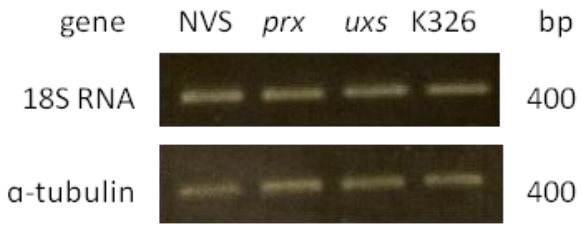

Figure 27 RT-PCR expression analysis of control gene 18S and α-tubulin, which is indicative of mycelial growth, in *Phanerochaete chrysosporium* grown on NVS, *prx,* K326 and *uxs* acetone insoluble cell wall material (AIM). The expression of α-tubulin is relative to 18S is constant across *P. chrysosporium* grown on all four substrates. Image is representative of three biological replicates.

4.4 Pretreatment with *Phanerochaete chrysosporium* causes lignin depolymerisation and loss of carbohydrate

Lignin content of acetone insoluble cell wall material was reduced after pretreatment (Table 8) confirming that *Phanerochaete chrysosporium* depolymerises lignin. After 10 days incubation with 10 mg AIM, the fungus had stripped more than 46% of lignin from every line. NVS lignin was the least affected, so the fungus appears to be able to depolymerise and metabolise modified cell wall material. In addition to reduced lignin content, *ccr* and *prx* have altered syringyl/guicyl ratios. NVS has G/C ratio of 0.82, while *ccr* and *prx* have higher S levels, the ratios being 1.64 and 1.21 respectively (Cook et al., 2011; Blee et al., 2001; O'Connell et al., 2002).

In addition to depolymerising lignin in AIM, *P. chrysosporium* hydrolyses cell wall polysaccharides (Sharay et al., 2008; Hammel et al., 2002). The reduction in total carbohydrates in the cell wall of *ccr* and *c4h* lines was ten-fold more than the loss of carbohydrates from NVS cell walls. As seen in Table 8, *prx* cell wall material (AIM) lost 3.5% of sugars. Lines *ccr* and *c4h* had 10.5% and 12% reduction respectively.

Polysaccharides in wildtype line K326 suffered less degradation than those in *uxs*, while lignin depolymerisation was not significantly different between the two lines. This is consistent with the data from the lignin down-regulated lines; the reduction of xylan or lignin had a positive effect on fungal degradation of the cell wall; where lignin synthesis was inhibited, *P. chrysosporium* was able to access and hydrolyse more lignin and where xylan synthesis was suppressed, polysaccharide depolymerisation increased.

Table 8 Percentage reduction in biomass of cell wall material after 10 days of fungal pretreatment as compared to cell wall samples that were treated with water. Data given are representative of three biological replicates, each with two technical replicates. Standard deviation is shown.

Line	% reduction in carbohydrate content	% reduction in lignin content
WT(NVS)	1.7 ± 0.9	46.41 ± 4.5
Prx	3.6 ± 0.7	80.85 ± 3.6
Ccr	10.7 ± 0.6	82.15 ± 2.3
c4h	12.1 ± 0.5	74.82 ± 6.6
WT(K326)	7.3 ± 1.1	70.11 ± 6.2
uxs	9.0 ± 0.6	65.03 ± 10.5

4.5 Pretreatment with *Phanerochaete chrysosporium* improves saccharification of stem AIM

Pretreating acetone insoluble cell wall material (AIM) with *P. chrysosporium* improved saccharification of all the tobacco lines (Figure 28). This was due to the reduction in lignin content seen in pretreated AIM (Table 8) and confirmed that lignin is the main barrier to efficient sugar release from cell walls.

Fungal pretreatment exposed 82% of sugars in *prx* cell walls to cellulolytic enzymes, compared to 58% which were exposed in non-pretreated walls (Table 8). This significant improvement was seen in *ccr* as well; the proportion of total sugars released from *ccr* AIM doubled after pretreatment from 20% to 43%. The improvement was still more impressive in K326, in which the percentage of theoretical yield of carbohydrates released enzymatically nearly triples.

Sugar release by enzymatic saccharification was from the acetone insoluble cell wall material (AIM) not from the fungal mycelium, which cannot be separated from the AIM after pretreatment. This was demonstrated by saccharifiying fungal mycelium grown on a plate. The results are shown in Figure 28B, and were the same as the sugars release from the enzyme control. This is expected as fungal cell walls do not contain the same polysaccharides as plant cell walls so the cellulases, xylanases and pectinases in the enzyme cocktail did not hydrolyse them.

In real terms, the saccharification profile of the tobacco lines was unchanged after pretreatment. Although more sugar was released, *prx* remained the line with the most

sugar released per mg AIM. Conversely the slight increase in sugar release from *uxs* as compared to the wildtype K326 was reversed after fungal pretreatment. K326 showed the biggest percentage increase in sugar release, releasing significantly more sugar than *uxs* after pretreatment.

There are examples of larger improvements in sugar release in the literature. Cellulase treatment of rice straw liberated 20% of the theoretical yield before pretreatment, but after incubation with *Phanerochaete* 55% of total carbohydrates could be released (Bak et al. 2009). The percentage of total cell wall carbohydrates released from wheat straw was three times greater after pretreatment (Zeng et al., 2010; Hatakka, 1983). The increase in sugar release seen here is nevertheless significant, especially given that 82% of wall carbohydrates were released from *prx* AIM by saccharification following pretreatment.

Improvement in sugar release per mg of AIM was not proportional to the percentage of lignin depolymerised by *P. chrysosporium* during the 10 days of pretreatment (Table 8). Lignification peroxidase down-regulated line *prx* had the same high reduction in lignin content as *ccr* and *c4h* after pretreatment, at 81%, but had the lowest improvement in sugar release. Xylan down-regulated line *uxs* had the same extent of lignin depolymerisation as the wildtype K326, but had 1.82-fold increase in sugar release compared to 3.02-fold improvement in K326.

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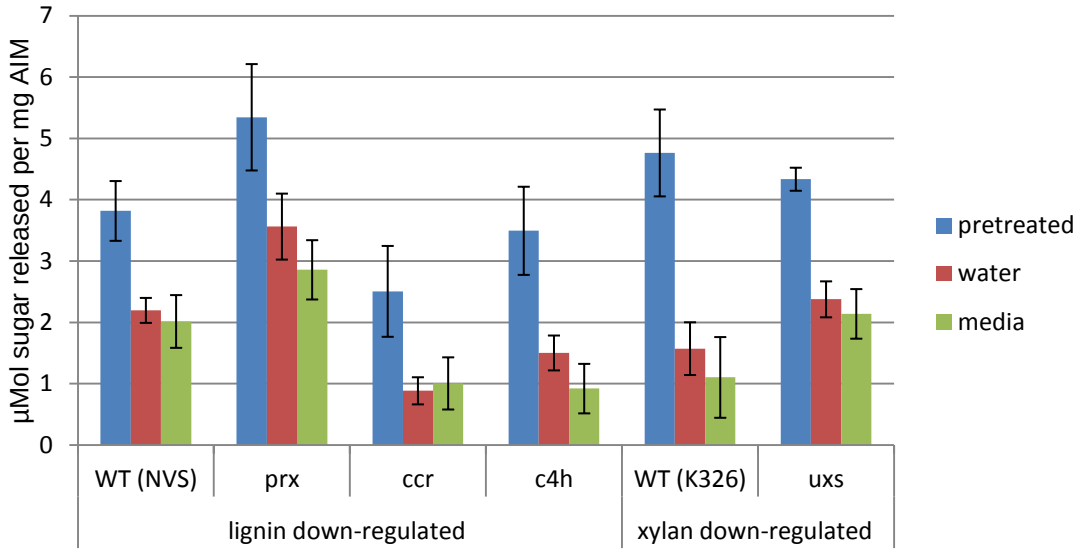

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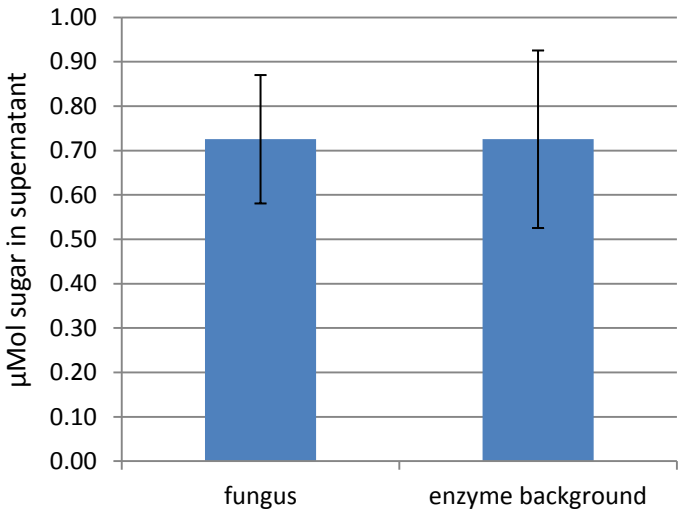

Figure 28 Improvement in sugar release from cell walls after pretreatment with *P. chrysosporium*

A Sugars released by saccharification of AIM after 10 days incubation with *Phanerochaete chrysosporium*, the culture media with no fungus, or water.

B Sugars present in the supernatant after saccharification of fungal mycelium, and after incubating the enzymes with no substrate under the same conditions. The 'enzyme background' absorbance reading was subtracted from the original absorbance from every result shown in (A) prior to conversion to µMol.

Data given are representative of five biological replicates, each with three or four technical replicates. Error bars represent standard deviation.

Table 9 Comparison of consequences of pretreatment on saccharification efficiency, reduction of lignin content and improvement in sugar release. Data representative of five biological replicates, each with three technical replicates.

Line	% of theoretical yield released by enzymes			% reduction in lignin content	Fold change improvement in sugar release
	Fungal pretreatment	No pretreatment	Media control		
NVS	42.6	32.6	29.9	46.41 ± 4.5	1.73
prx	82.3	58.4	57.0	80.85 ± 3.6	1.5
ccr	43.3	19.8	24.0	82.15 ± 2.3	2.83
c4h	34.3	19.3	16.9	74.82 ± 6.6	2.32
K326	33.7	13.6	15.0	70.11 ± 6.2	3.02
uxs	37.3	25.1	22.1	65.03 ± 10.5	1.82

4.6 Enzymatic saccharification of pretreated AIM releases glucose and xylose

GC-MS analysis of the sugars released by saccharification of AIM pretreated with *Phanerochaete chrysosporium* suggested that only cellulose and xylan were hydrolysed by the enzymes (Table 10). Only glucose and xylose were present in the hydrosylate after incubation. The control samples also contained just glucose and xylose. This is compared to uronic acid, rhamnose, arabinose, xylose, mannose and glucose which were released from non-pretreated cell wall material (B

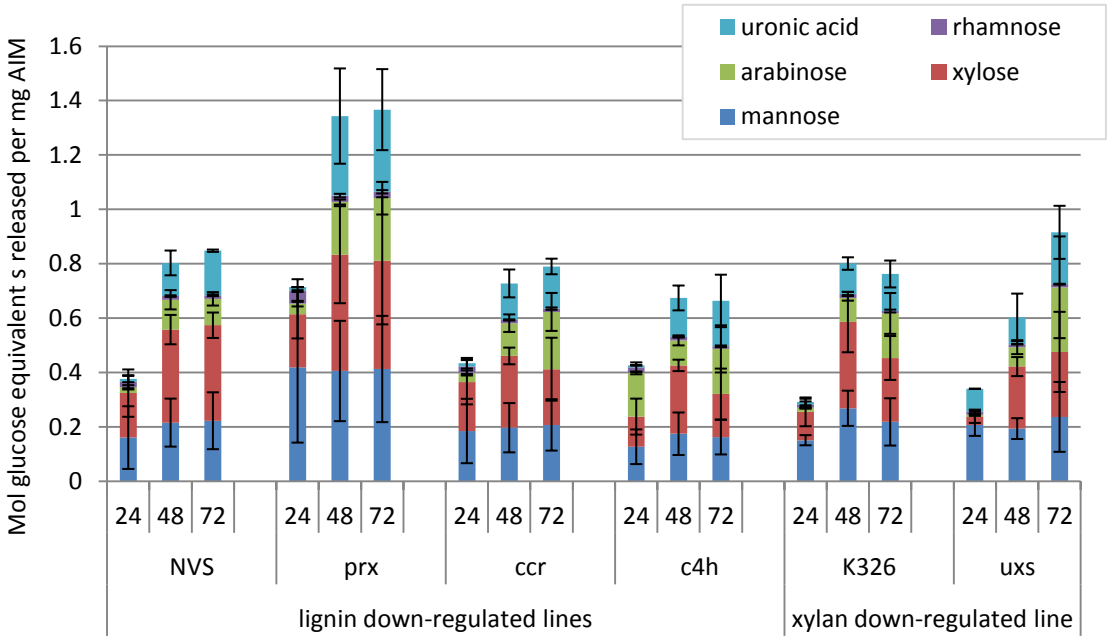

Figure 23). GC-MS profiles of the enzyme background were the same in both analyses, demonstrating that the differences in sugar release were caused by pretreatment rather than the enzymatic saccharification.

The recalcitrance of crystalline cellulose ensures that it is not hydrolysed by the preparation of the samples after incubation with for ten days before they are subjected to saccharification. However it appears that the protocol causes pectin and hemicellulose to be degraded as less than 3% of the sugars released are non-cellulosic sugars. Before the pretreatment, which includes incubation at 37°C for 10 days either with *P. chrysosporium* or in the control tubes, the samples are heated at 100°C for 15 minutes in order to sterilise them. After incubation, in which it is possible the osmotic pressure cause the cell walls to expand, it is washed with 80% acetone and again boiled to ensure the fungus is dead and will not interfere with saccharification.

Table 10 Percentage monosaccharide composition of sugars released from AIM by enzymatic saccharification after 10 days pretreatment. Sugars released by enzymatic saccharification after pretreatment with *P. chrysosporium* or incubated with control medium are comprised of more than 97% glucose. Data is the average of two biological replicates, each with two technical replicates,

Treatment	**sugar**	**% composition in cell wall modified lines**					
		NVS	*ccr*	*c4h*	*prx*	K326	*Uxs*
Fungus	glucose	100.0	98.6	98.4	99.8	97.2	97.4
	xylose	0.0	1.4	2.2	0.5	2.8	2.6

Media control	glucose	100.0	97.5	99.1	98.8	98.0	97.8
	xylose	0.0	2.5	0.9	1.5	2.0	2.2
water	glucose	100.0	97.5	99.6	99.7	98.4	96.8
	xylose	0.0	3.3	0.4	0.4	2.3	2.2

4.7 Pretreatment of primary cell wall modified lines also improves saccharification in *Arabidopsis*

Section 4.5 demonstrated that lignin depolymerisation by *Phanerochaete chrysosporium* is an effective way of improving the saccharification properties of tobacco lines, including material in which the secondary cell wall had been modified. The next logical step was to investigate whether fungal pretreatment had the same effect on transgenic plants in which the primary cell wall had been targeted. The primary cell wall modified plants used were *Arabidopsis thaliana* lines (kindly given by Felice Cerevone, Università di Roma la Sapienza). The transgenic Arabidopsis lines have cell walls with modified pectin and are described in Section 2.3.6.

Arabidopsis stems, like tobacco, have a lignin content of about 20% (Bhargava et al., 2010). It is not surprising then that pretreatment of *A. thaliana* acetone insoluble material (AIM) with *Phanerochaete chrysosporium* made the AIM more amenable to cellulolytic hydrolysis than AIM that has not been pretreated (Figure 29).

There was a difference in saccharification of unpretreated AIM from Col-0 and the *pg* lines. The improvement in sugar release from fresh tissue has previously been described (Lionetti et al., 2009) and is due to the reduced pectin content in *pg57* and *pg27* lines, described in Capodicasa et al. (2004), preventing access of lignin peroxidase to the lignin. These lines express an *Aspergillus niger POLYGALACTURONASE II* gene, which resulted in increased pectin degradation by enhancing polygalacturonase (PG) activity. Consequentially *pg* lines have 25% less pectin than wildtype.

Pectin down-regulation appeared to have two opposite effects on lignin metabolism by *P. chrysosporium*. Saccharification of lines in which pectin is degraded by an endogenous polygalacutonase, *pg57*, was not improved by pretreatment. However, plants with reduced pectin methyl esterase (PME) activity, *pmei2*, showed significant

improvement in saccharification after pretreatment with *P. chrysosporium* ($p = 2.82 \times 10^{-4}$ according to the Student's T-test). *pmei* lines have reduced quantities of long chains of unesterified homogalacturonan, and higher degrees of homogalacturonan methylesterification.

It has been suggested that when the cell has finished elongating, PMEs enhance the formation of benzyl-uronate cross-links in lignin (Mohnen, 2008). PMEs are unaffected in *pg57*, but in *pmei2* PME activity is inhibited. It is possible that the loss of PME mediated cross-links allowed more lignin depolymerisation in this line than in the wildtype or in *pg27* or *pg57*. Conversely reducing pectin content by over-expressing PG appears to reduce accessibility of lignin peroxidase to lignin.

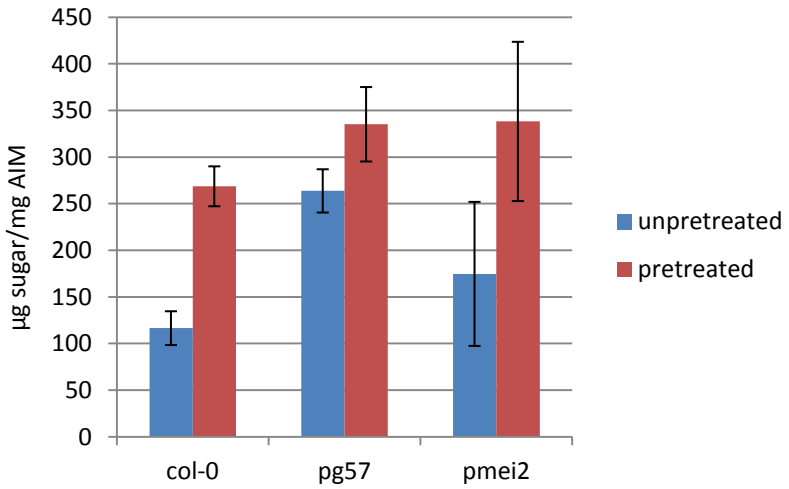

Figure 29 Improvement in sugar release from cell walls of Col-0 and *pmei* after pretreatment with *P. chrysosporium*. Saccharification of AIM after 10 days incubation with *Phanerochaete chrysosporium* or 10 days incubation with water. Data is the average of two biological replicates, each with three technical replicates. Error bars represent standard error.

4.8 Pretreatment of fresh *Arabidopsis thaliana* (Col-0) stems with *Phanerochaete chrysosporium*

Having established the efficacy of *Phanerochaete chrysosporium* pretreatment on tobacco and Arabidopsis stem acetone insoluble material (AIM), secondary cell wall modified AIM and primary cell wall modified AIM, the next step was to test its effect on fresh stem material. As seen in Figure 30, *Phanerochaete chrysosporium* pretreatment was effective at exposing cellulose in fresh stem material. Sugar release from fresh Arabidopsis stem material increased 4.6-fold after one week of pretreatment.

Sugar release increased fourfold after pretreatment. This scale of improvement is higher than seen in published research which used fresh material as the substrate, which range from 3.5-fold improvement in pretreated wheat straw (Zeng et al., 2010) to 2.5-fold in rice straw (Bak et al., 2009).

These data show pretreatment of fresh biomass was more effective than pretreatment of cell wall material when it comes to improving sugar release. Pretreatment of AIM improved sugar release up to three fold in wildtype K326 but less than twofold in wildtype NVS, *prx* and *uxs.* This may be because fresh material is the natural substrate for *Phanerochaete chrysosporium*, which naturally grows on the trunks of trees.

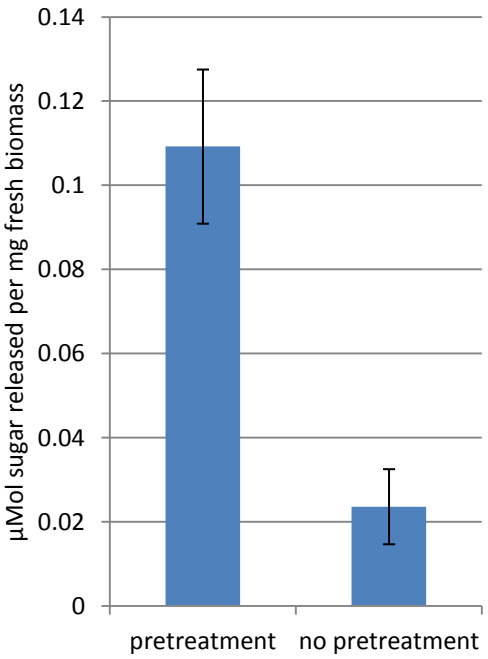

Figure 30 Sugar release from Arabidopsis stem material with and without pretreatment with *Phanerochaete chrysosporium.* Pretreatment improved sugar

release from fresh stem material. Data is from three biological replicates, each with three technical replicates. Error bars represent standard deviation.

4.9 Discussion

The principal conclusion from this chapter is that *Phanerochaete chrysosporium* pretreatment is effective at improving the saccharification efficiency of cell walls engineered for down-regulated lignin and hemicellulose. In addition, cell wall modifications significantly increase lignin depolymerisation by *P. chrysosporium*. This suggests that the lignin peroxidase secreted by the fungus is not highly specific but is able to act on lignin cell walls that differ from the wildtype in lignin content and composition.

Compared to the results available in the literature, a 60% reduction in lignin content as seen in Table 8 is a considerable achievement. Pretreatment with *P. chrysosporium* resulted in lignin reduction of 15% in rice straw after 15 days treatment (Bak et al., 2008), 20% in cotton stalk after 12 days treatment (Shi et al., 2009) and 26% in wheat straw after one week of treatment (Zeng et al., 2010). Similarly, the level of polysaccharide hydrolysis by *P. chrysosporium* is less than seen in previous investigations. Pretreated rice straw gave 30% cellulose recovery compared to 38% prior to pretreatment (Bak et al., 2007). There was 23% loss of cellulose in wheat straw after pretreatment (Salvachúa et al., 2011), but a different method gave 36% cellulose reduction after pretreatment of wheat straw (Zeng et al., 2010).

The research described above used varied protocols to get results. The methods used differ from the method presented in this project in that the substrate was fresh material, usually ground. Here, acetone insoluble cell wall material (AIM) was pretreated. Additionally none of the research used veratryl alcohol in the culture medium, and only Bak et al. (2009) added extra carbon in the form of glucose to the culture medium. It is worth noting that also cotton stalk contains 29% lignin (Shi et al., 2009), while tobacco as well as wheat and corn straw have an average lignin content of 20% so results from cotton straw may not be as comparable to the data presented in this chapter as results from wheat and corn. The addition of veratryl alcohol appears to be the principal reason for the extent of lignin depolymerisation, as seen in Figure 25 and Figure 26. The addition of veratryl alcohol, as recommended in Tien and Kirk (1988), caused both improved saccharification and lignin reduction in pretreated AIM.

The benefits of *P. chrysosporium* pretreatment on saccharification have already been tentatively established (Zeng et al., 2010; Shestha et al., 2007; Bak et al., 2007; Hatakka et al., 1983). However this is the first study in which plants with modified cell walls were used as a substrate. It demonstrates that by combining lignin down-regulation and fungal pretreatment, it is possible to release 82% sugar from cell walls. Although this is the first study on tobacco, this figure is higher than any previous studies. Pretreated rice straw yielded 55% of total carbohydrates (Bak et al., 2007) while in wheat straw 35% of the theoretical yield was converted to monosaccharides after pretreatment.

There is no obvious correlation between percentage reduction in lignin content and percentage improvement in saccharification. The rate of lignin depolymerisation was highest in *ccr* and *prx*, and then in *c4h* with the wildtype NVS being the line which had the least reduction in lignin. However improvement in sugar release after pretreatment was smallest in *prx.* When comparing the sugar released per mg of AIM and the percentage of total cell wall carbohydrates released, it is obvious that *prx* did not fit the trend that improvement in sugar release is likely to be correlated with the reduction of lignin content. NVS, *ccr* and *c4h* fit this correlation. There was less carbohydrate in *prx* cell walls, so the release of sugar is close to the maximum. 82% of the theoretical yield of sugars was released from *prx*, while less than 45% was released from NVS, *ccr* and *c4h*. The *prx line* also showed high saccharification efficiency (Figure 22) due to a significantly reduced lignin content (Table 6) and further improvement was probably limited. However the trend is clear: depolymerisation of lignin improves sugar release from cell wall material by enzymatic saccharification.

P. chrysosporium strains vary with regards to lignin peroxidase activity and preferred substrate (Blanchette et al., 1992). In the research presented here, it appears that the preferred substrate of the particular strain of *P. chrysosporium* utilised is lignin, as lignin depolymerisation is high compared to published research while polysaccharide hydrolysis is low.

Line *uxs* had the same proportion of lignin depolymerised as the K326 wildtype, but did not show a comparable improvement in sugar release. This was due to the higher rate of saccharification before pretreatment, the increased lignin content (Table 6) and the reduction in cell wall carbohydrate caused by interrupting xylan biosynthesis and the lack of compensatory increased cellulose deposition. There were fewer sugars to release in *uxs* and more lignin to depolymerise. It is possible that a longer pretreatment time in which more lignin is depolymerised would improve sugar release from *uxs*.

The versatility of *P. chrysosporium* is demonstrated in this chapter. It significantly improveed saccharification of tobacco cell wall material, Arabidopsis cell wall material and fresh Arabidopsis stems. This may be more important industrially than improvement in saccharification due to cell wall modification, as use of genetically engineered plants is still restricted and mistrusted by the public.

It is evident that pretreatment of cell wall material with *P. chrysosporium* depolymerised lignin and improved cellulose accessibility and hydrolysis. However, the pretreatment protocol, rather than pretreatment with *P. chrysosporium*, is apparently responsible for the degradation of hemicelluloses and pectins, as the sugars released from cell walls after the control pretreatment are the same as those from fungal pretreatment, and do not contain non-cellulosic sugars. Some explanations for this are presented below. Additionally, exposing lignocellulosic material to hot water or very high temperatures can hydrolyse hemicellulose and pectin (Mosier et al., 2005; Hendricks et al., 2009). Though these polysaccharides usually need temperatures of more than 150°C before degradation occurs, it is possible that the additional treatments to AIM, including long incubations with water following washes with acetone, phenol and chloroform, favour hydrolysis at lower temperatures.

Cellulases and other cell wall carbohydrate degrading enzymes adsorb to lignin, becoming denatured or else inhibited by the non-productive binding (Rahikainen et al., 2011). It is possible that the pretreatment protocol, for the reasons described above, increased the lignin surface area exposed to the enzymes. More sugars were released after pretreatment, but perhaps the almost total absence of non-cellulosic sugars in the saccharification supernatant reflects the limited amount of xylanases and pectinases in the cellulolytic enzyme cocktail in addition to the larger amount of cellulose compared to non-cellulosic carbohydrates present in the cell wall. The ratio of cellulosic to non-cellulosic polysaccharides may have increased during the pretreatment protocol, as xylans and pectins are more amenable to hydrolysis due to physical pretreatment. Additionally, increased non-productive binding to lignin that may have occurred would affect the limited number of xylanases and pectinases more than cellulases, which were in abundance. The occurrence of both these events would cause the lack of non-cellulosic monosaccharides released by saccharification.

While this is not of great industrial importance, as glucose is the commercially valuable monosaccharide, complete understanding of this new protocol requires identification of the point at which these polymers are degraded.

5 Identifying novel genes involved in cell wall synthesis

5.1 Introduction

A xylogenic tobacco cell culture was derived from tobacco transformed with the *Tcyt* gene from *Agrobacterium* (Blee et al*.,* 2001). The *Tcyt* gene enhances high endogenous levels of cytokinin. Cells form tracheids in culture. RNA was isolated from this culture and converted into cDNA. The cDNA sequences were ligated into the λZAP II UniZAP-XR vector and finally inserted into pBluescript vector (both vectors from Stratagene; Agilent Technologies Inc., Santa Clara, CA, USA) and transformed into *E. coli*. Of this library, 3000 colonies were kept in glycerol stocks. Fragments of cDNA were amplified and sequenced, creating database of Expressed Sequence Tags (ESTs). 2668 genes are represented in the EST database (Cook et al., 2011), which is stored in Genbank (EH663598 – EH666265).

Bioinformatics is an invaluable first tool for placing genes in pathways and networks and identifying which genes and stimuli may affect their regulation. Here, the EST database from the xylogenic culture was mined for transcription factors and cell wall synthesis genes. The EST sequences were used to design primers to analyse transcript levels of genes in the lignin, cellulose and xylan biosynthesis pathways. Additionally, online resources such as Genemania (Warde-Farley et al., 2010) and Genevestigator (Hruz et al., 2008) were utilised to select genes with potential involvement in regulation of cell wall synthesis.

5.2 Analysis of secondary cell wall synthesis genes in xylogenic cell cultures

The EST database analysed contained 2668 genes. The ESTs were annotated according to BLAST hits from the NCBI BLASTn database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), as of April 2011. 357 (13%) were not significantly similar to any genes. Of the remaining EST sequences, 313 (16% of the whole database) were aligned to unknown proteins and 185 (7%) were most similar to clones largely from the unannotated genomes of tomato, tobacco and poplar. These

498 genes represent the large gap in current knowledge of cell wall biosynthesis and modification.

The data demonstrate that the Tcyt-derived EST library contained 13% (223) cell wall related genes (Cook et al., 2011), showing the extent of secondary cell wall synthesis enrichment in xylem. These genes are listed in Table 11.

5.2.1 Cellulose synthesis genes

Cellulose is synthesised using UDP-glucose. Two possible source of UDP-glucose are through the breakdown of sucrose into glucose (Haigler et al., 2001) or the hydrolysis of starch, as starch grains disappear rapidly in these tissues during secondary wall formation (Bolwell, 1993). Transcripts were present for genes encoding enzymes necessary for both starch and sucrose hydrolysis (Supplementary data I, Table 11): *SUCROSE SYNTHASE* (*SUSY*; EH664527, EH664745, EH664820, EH664193 EH666077, EH666169, EH666255) which converts sucrose into UDP-glucose and fructose, and three invertases (EH664106, EH664540 EH665657) which cleaves sucrose into two monosaccharides (reviewed by Sturm and Tang, 1999); and *UDP-GLUCOSE PYROPHOSPHORYLASE* (reviewed by Kleczkowski et al., 2004; EH664780, EH664052, EH664554, EH664604, EH664969, EH665027, EH665911, EH666132, EH663806). *AMYLASE* (EH663749) which hydrolyses starch was also represented. If EST abundance is a true reflection of elevated gene expression this could be related to the increased production of cellulose during secondary wall formation.

There were three *CELLULOSE SYNTHASE* genes in the EST library (EH663724; EH663943, EH664994). In other systems, *CELLULOSE SYNTHASE-LIKE* (*CSL*) and *CELLULOSE SYNTHASE* (*CESA*) genes are usually below 0.03% in ESTs (G.P. Bolwell, personal communication), so the low abundance seen may be consistent with other reports.

5.2.2 Lignin synthesis genes

Genes specific for the lignification pathway leading to monolignol synthesis are present in our list. Surprisingly, no ESTs coding for *PHENYLALANINE AMMONIA-LYASE* (*PAL*) appeared, however two different class I *CINNAMATE 4-HYDROXYLASES* (*C4H*) were found (EH664914, EH665327) probably representing the two isoforms in the allotetraploid, *Nicotiana tabacum*.

EH663728 is annotated as *COUMAROYL-ESTER-3-HYDROXYLASE* (*C3H*) which catalyses the next hydroxylation step in the pathway after cinnamate 4-hydroxylation. Representatives of the rest of the pathway, *CAFFEOYL-COA METHYL-TRANSFERASE* (*CCOMT*; EH665253, EH665876), *CAFFEIC ACID O-METHYL TRANSFERASE* (*COMT*; EH665400, EH665510, EH666153, EH663855), *CINNAMOYL-COA REDUCTASE* (*CCR*; EH664240, EH664699, EH666151, EH666260), *CINNAMYL ALCOHOL DEHYDROGENASE* (*CAD*; EH664137, EH664150, EH664162, EH664196, EH664225, EH664374, EH664909) and *HYDROXYCINNAMOYL-COA:QUINATE HYDROXYCINNAMOYL TRANSFERASE* (*HCT*; EH664996, EH666193) are present on the list. These include genes targeted in a number of antisense down-regulation programmes for each of these reactions (Anterola and Lewis, 2002). The only major absent ESTs were for *4- COUMARATE LIGASE* (*4CL*) and *PAL*, however their expression was detected by RT-PCR in xylem tissue of wild type tobacco (see Section 5.5).

5.2.3 Gene expression and matrix polysaccharide formation

To dissect the relationship between the various components of the cell wall, it is important not to focus only on the effects of the manipulation of the lignin biosynthesis pathway but to understand the synthesis and regulation of other non-cellulosic polymers in secondary walls of dicots such as xylan. Isolation of wall proteins involved in modification and possible assembly of secondary wall xylan can also underpin future development of engineering plant fibre.

Glucuronoarabinoxylan synthesis is, along with lignin, a target for cell wall manipulation. The specific vascular genes required for the provision of the substrates UDP-xylose and UDP-glucuronate have been identified (Bindschedler et al., 2005; Bindschedler et al., 2007) and were all represented in our ESTs collection: *ADH-LIKE UDP-GLUCOSE DEHYDROGENASE* (*ADH*; 20 ESTs), *UDP-GLUCOSE 6-DEHYDROGENASE* (*UGD*; EH663670, EH665796), *UDP-GLUCURONATE DECARBOXYLASE* (*UXS*; EH663981, EH664621, EH664838, EH664948) and *UDP-GLUCURONATE 4- EPIMERASE* (*UG4E*; EH664555).

Xylan biosynthesis is followed by assembly and possibly remodelling. Therefore in addition to xylan synthase, secondary wall xylanase and xylan binding protein may be required. In support to this, two full length xylanase clone cDNAs (TQ152919 and DQ152919) were also obtained from the EST library (Cook et al., 2011).

Table 11 Summary of ESTs in the database representing genes involved in synthesis or modification of cell wall components.

Function	Number of genes	Function	Number of genes
Lignification		Polysaccharide biosynthesis	
Cinnamic acid 4-hydroxylase (C4H)	2	UDP-glucuronate decarboxylase (UXS)	4
Cinnamoyl CoA reductase (CCR)	4	ADH-like UDP-glucose dehydrogenase (ADH)	20
Cinnamyl alcohol dehydrogenase (CAD)	7	UDP-glucose 6-dehydrogenase (UGD)	2
Catechol O-methyltransferase (COMT)	4	UDP-glucuronate 4-epimerase (UG4E)	1
Caffeoyl CoA O-methyltransferase (CCOMT)	2	Cellulose synthase-like (CSL; homologues of *A. thaliana* CSLA09 and CSLD3)	2
4-coumarate 4-hydroxylase (C3H)	1	Glucosyltranferase (unknown tobacco GT)	1
Peroxidase	27	Glycosyltransferase (homologues of *A. thaliana* GAUT8 and GT14 (x2), *Polulus tremula* GT14, and *Lycopersicon esculentum* GT8)	5
Cell wall modifying proteins		Xylosyl transferase	1
Pectinesterase	8	Sucrose synthase (SUSY)	7
Extensin	3	Invertase	3
Proline-rich protein	5	Amylase	1
Glycine-rich protein	5	UDP- /ADP-glucose pyrophosphorylase	11
Expansin	7	Cellulose synthase (CESA)	3
Pectin methylesterase	7	Transcription factors	
Xyloglucan endotransglycosylase	8	Total	70

5.3 Effects of *CCR*, *C4H*, *PRX* and *UXS* down-regulation on vascular specific transcription factors

5.3.1 Identification of xylem specific transcription factors

In order to identify cell wall synthesis regulators, the transcription factors present in the EST library were analysed using online bioinformatic tools to profile their expression. There were 70 transcription factors represented in the EST library from the Tcyt tobacco cell culture (Table 11).

The accession numbers of the Arabidopsis homologues of 70 transcription factors in the EST database were extracted by searching for terms using the find function in Microsoft Excel. The terms searched were: transcription; zinc finger; zinc knuckle; DNA-binding; and leucine zipper. The accession numbers were entered into Genevestigator (Hruz et al., 2008). As the *in silico* expression analysis tools are limited for the recently sequenced tobacco genome, Arabidopsis homologues of the transcription factors were used. The Genevestigator anatomy expression profile output is shown in Figure 31 and Table 12. *SVP* (*SHORT VEGETATIVE PHASE*)*, RAP2* (*RELATED TO APATELA 2*)*, REV (REVOLUTA)* and *RSZ33* (*ARGININE/SERINE-RICH ZINC KNUCKLE-CONTAINING PROTEIN 33)* are all highly, though not uniquely, expressed in the xylem. They have all been linked to regulation of inflorescence or more generally meristem specification.

SVP is expressed in the stem at all stages of Arabidopsis growth (TAIR) but has no known function in xylogenesis. It is however essential for the initiation of the floral meristem (Gregis et al., 2008), and for the development of normal flowers as it recruits *AGAMOUS1* repressors *LEUNIG* and *SUESS* (Gregis et al*.,* 2006).

REV is expressed in the very early stages of floral and lateral meristem development, perhaps initiating meristem regulators. Significantly for this study, it is thought to be involved in positioning or development of xylem tissue. Class II LD/ZIP family members, which include *REV*, often have gene expression limited to the developing xylem. Mutants of *REV* itself have abnormal vascular structure. The *REV* over-expresser *rev-10* has xylem vessels surrounding central phloem cells rather than the normal peripheral phloem surrounding central xylem vessels (Emery et al., 2003). *rev* mutants have thickened stems and extra cell layers in the cambium (Talbert et al., 1995).

Table 12 Mean signal value of transcription factors expressed highly in the xylem. Output is from the anatomy expression profile from Genevestigator (Hruz et al., 2008).

		Tissue type						
AGI number	**Gene name**	Callus	Cell culture	Xylem	Seedling	Inflorescence	Rosette	Roots
AT1G53910	*RELATED TO APETELA 2.12 (RAP2.12)*	7607	13353	21937	10463	12070	8602	13997
AT2G22540	*SHORT VEGETATIVE PHASE (SVP)*	1770	1035	7582	1391	1031	2923	1059
AT2G37340	*RSZ33*	10913	9550	21162	3774	7057	3052	5423
AT5G60690	*REVOLUTA (REV)*	26705	13926	27121	10299	12596	8663	15174

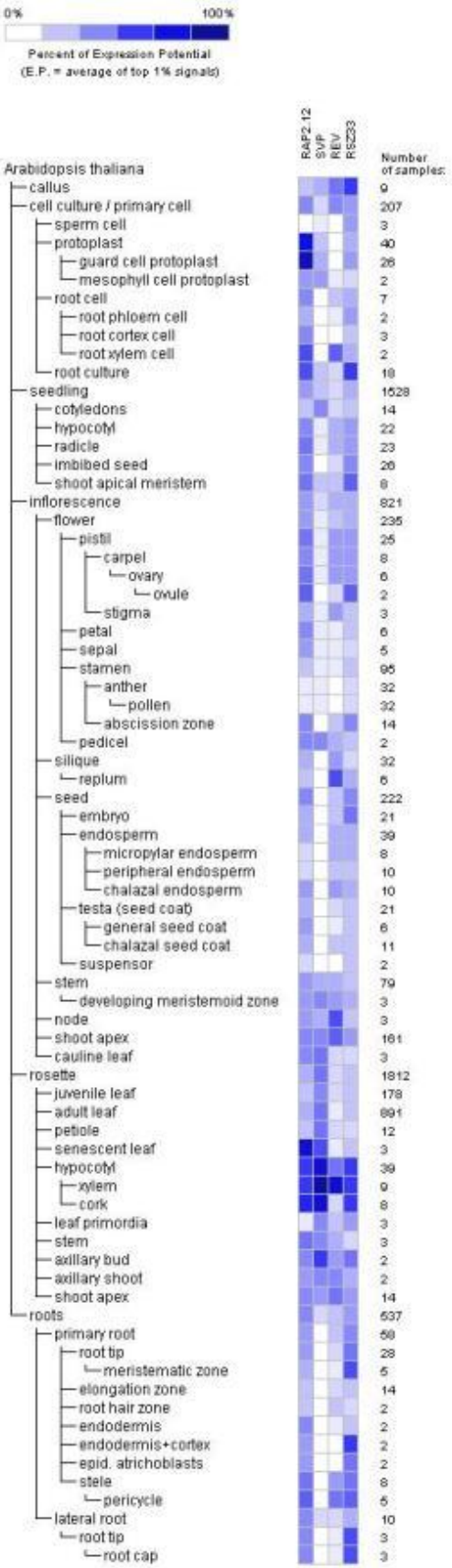

Figure 31 Anatomy expression profile showing expression of *RAP2.12, SVP, REV* and *RSZ33* in different plant tissues. Output from Genevestigator (Hruz et al. 2008)

shows the level in expression in intensity of colour. There is high expression in xylem tissue. *REV, REVOLUTA; SVP, SHORT VEGETATIVE PHASE; RAP2, RELATED TO APATELA 2.12; RSZ33, ARGININE/SERINE-RICH ZINC KNUCKLE-CONTAINING PROTEIN 33.*

RZS33 is so named because it contains two zinc knuckles in an arginine/serine rich domain. It is a phosphoprotein which is expressed at a low level indiscriminately in Arabidopsis, but has high expression in the root and hypcotyl xylem (Figure 31; Hruz et al., 2008). Lopoto et al. (2002) proposed that RSZ33 has a role in spliceosome assembly, as argenine/serine rich proteins commonly play a crucial role in splicing and RSZ33 interacted with other Arabidopsis splicing factors in a yeast two-hybrid screen. Kalyna et al. (2003) confirmed its involvement in splicing by observing abnormal splicing patterns in *RSZ33* pre-mRNA and also in three other genes (*atSRP30, atSRP34/SRI* and atRSp31) in transgenic Arabidopsis *RSZ33* over-expressers (Kalyna et al., 2003; 2006). These plants also showed unusual phenotypes including atypical embryogenesis and seedling development. Abnormal polarization of cell division, greater cell expansion and up-regulation of *AIR3* which is involved in auxin-induced lateral root formation, were the effects of ectopic expression of *RSZ33*.

The protein family RAP2 is a large collection of proteins that all share a common domain of 432 amino acids. This sequence is called the AP2 domain as it was originally identified in APETALA2 and is essential for AP2 function (Jofuku et al., 1994). The EST sequence EH665196 had a highly significant alignment (1E-47) to a tobacco AP2-domain containing transcription factor second other alignment (1E-9) to the *Arabidopsis thaliana RAP2.12*. Although the functions of all the RAP2 family members are uncertain, it is known that they have a similar expression to *AP2*, not only being expressed during inflorescence initiation and specification but also in the stem and leaves, indicating that they have an effect on vegetative growth too (Okamuro et al., 1997). They are involved in the ethylene response (Lin et al., 2008; Jofuku et al., 1994) and in drought stress tolerance (Lin et al., 2008).

5.3.2 RAP2.12 and RSZ33 are differentially regulated in lignin down-regulated lines

REV did not show large transcriptional changes in any of the lines (Figure 32), but it was up-regulated nearly twofold in *prx*. *REV* is involved in xylogenesis at an undefined stage, and *REV* mutants have vascular modifications (Emery et al., 2003; Talbert et al.,

1995). Line *prx* has a collapsed xylem phenotype (Kavousi et al., 2010), and the up-regulation of *REVOLUTA* may be linked to the modified xylem.

RAP2.12 was down-regulated more than fourfold in all the lignin modified lines (Figure 32), and nearly twofold in *uxs. RSZ33* and *SVP* also showed about two fold decreases in expression in *ccr, c4h* and *prx* but are not changed in *uxs*. *RSZ33* and *SVP* have not been implicated in xylem biogenesis or secondary cell wall deposition.

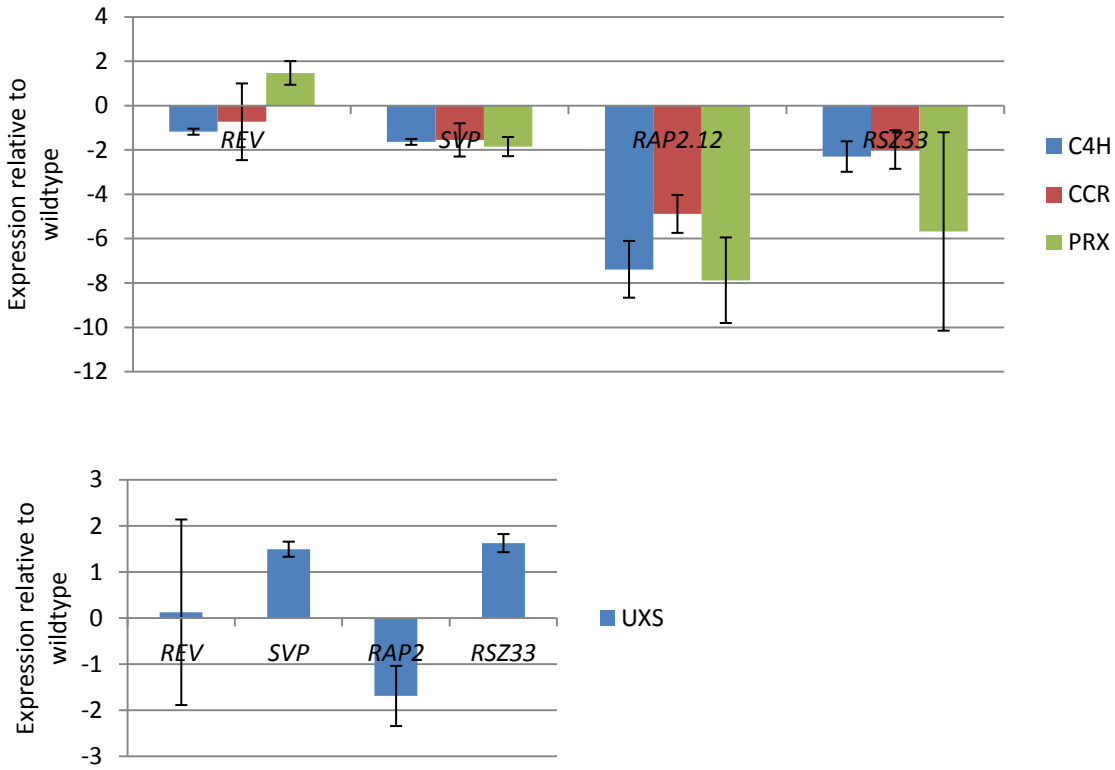

Figure 32 qRT-PCR expression analysis of transcription factors expressed in xylem tissue (Cook et al., 2011)**.** Data is given in terms of change in abundance of the transcript for each gene as compared to the wildtype, NVS for lines C4H, CCR and PRX, and wildtype line K326 for line UXS. Data are the average of 2 biological replicates, each containing 3 technical replicates. The bars indicate standard error. The reference gene was GAPDH.

REV, REVOLUTA; SVP, SHORT VEGETATIVE PHASE; RAP2, RELATED TO APATELA 2.12; RSZ33, ARGININE/SERINE-RICH ZINC KNUCKLE-CONTAINING PROTEIN 33.

5.4 Identification of potential novel cell wall regulator RAP2.12

5.4.1 RAP2.12 has similar expression pattern to cell wall synthesis genes

As *RAP2.12* showed the greatest change in expression of the xylem-specific transcription factors analysed in line *ccr, c4h, prx* and *uxs* by qRT-PCR (Figure 32), further *in silico* analysis was carried out to ascertain whether *RAP2.12* may be involved in cell wall synthesis regulation. Three separate online transcriptome analysis tools were used to analyze *in silico* the expression of *RAP2.12* alongside several cell wall synthesis genes. Genevestigator Metanalyzer (anatomy microarray dataset) (Hruz et al., 2008) allows visual display of expression data from different microarray experiments as a single heatmap. Tigr MultiExperiment Viewer (TMEV) performs hierarchical clustering (Saeed et al., 2006). Genemania (Warde-Ferley et al., 2010) allows identification of co-expression between genes.

The results from Genevestigator (Hruz et al., 2008) show that the expression levels of *4-CL, CAD6, CCOMT, CCR1, C3H, CESA3, HCT* and *PAL* as well as *RAP2.12* are comparable in all organs considered, decreasing homoneneously in siliques and inflorescence stem (Figure 33 B). Notably, *RAP2.12* has higher expression in the imbibed seed, ovule and root cap, in which cell wall synthesis genes have relatively low expression.

Tigr MultiExperiment Viewer (TMEV) hierarchical clustering tool was used to analyse the expression data of cell wall synthesis genes in different organs obtained in Genevestigator). *RAP2.12* clusters with hemicellulose synthesis genes *ADH, CSLD* and *UG4E* (Figure 33 A). This cluster has a common ancestor with that containing *CCR* and *CESA3, 4CL, CAD, CCOMT, HCT, PAL* and *C3H*.

Genemania (Warde-Farley et al., 2010) was used to obtain a possible co-expression network between RAP2.12 and other cell wall synthesis genes. Genes are represented by nodes and the grey edges identify the correlation between two genes. The correlation is derived from the expression profile of each gene across microarrays datasets. The results show that RAP2.12 is co-expressed with 4-CL1, HCT, C4H and CCR (AT1G15950;Figure 33C).These 4 lignin synthesis genes are also co-expressed with other genes involved in lignin biosynthesis. Unlike Genevestigator, the dataset for Genemania does not distinguish between tissue types. Moreover, the two databases contain different microarray datasets (Leivar et al., 2009; López-Martin et al., 2008;

Dohmann et al., 2008; Redman et al., 2004).. Therefore a direct comparison between the results obtained is not feasible.

However altogether these observations support and expand the conclusions that *RAP2.12* has similar expression levels to hemicellulose synthesis genes, as well as genes from the lignin biosynthesis pathway.

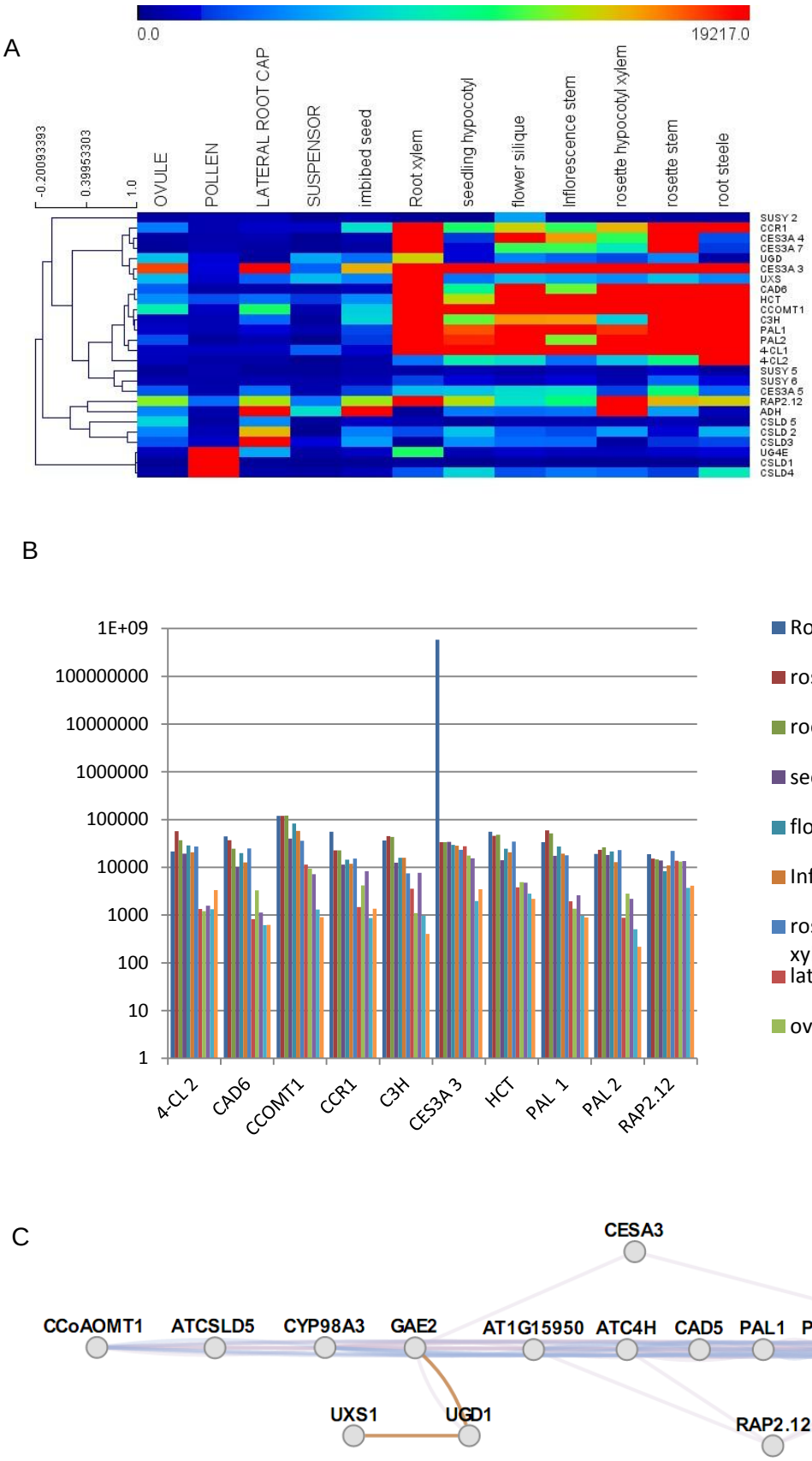

Figure 33 *RAP2.12* is coexpressed with cell wall synthesis genes. A Tigr MultiExperiment Viewer (TMEV) hierarchical clustering analysis of expression data of cell wall synthesis genes in different organs obtained in Genevestigator Metanalyzer

(anatomy microarray datasets). *RAP2.12* clusters with hemicellulose synthesis genes *ADH, CSLD* and *UG4E*. Data are from Arabidopsis databases. **B** Expression profiles of selected cell wall synthesis genes in different organs obtained in Genevestigator Metanalyzer (anatomy microarray datasets). The expression levels of all genes are comparable in all organs, decreasing homogeneously in siliques and inflorescence stem. **C** Co-expression network obtained from Genemania. Genes are represented by nodes and the grey edges identify the correlation between two genes. The correlation is derived from the expression profile of each gene across microarrays datasets. RAP2.12 is co-expressed with 4-CL1, HCT, C4H and CCR (AT1G15950)

5.4.2 Genotyping RAP2.12 insertion lines

Having established that RAP2.12 is co-expressed with cell wall synthesis genes and is significantly down-regulated in lignin suppressed lines *prx, ccr* and *c4h*, the next step was to investigate the effects of *RAP2.12* suppression on cell wall synthesis genes. Two RAP2.12 insertion lines were ordered: SALK _152421 (RAP SK; Alonso et al., 2003) and SAIL_1215 (RAP SL; Sessions et al., 2002). Before gene expression analysis, a number of plants were genotyped to ensure the lines analysed were homogygous. The genotyping results are shown in Figure 34; RAP SK plants 1, 3, 7 and RAP SL plants 2, 6, 9 are homozygous and were selected for transcriptome analysis. In RAP SK plant 7 and RAP SL plants 2 and 6, the insertion was in different orientations in the two chromosomes so a product was amplified by the use of the left border primer (LB or LBa1) and the primer up and downstream of the insertion (RAP2.12_R or RAP2.12_L).

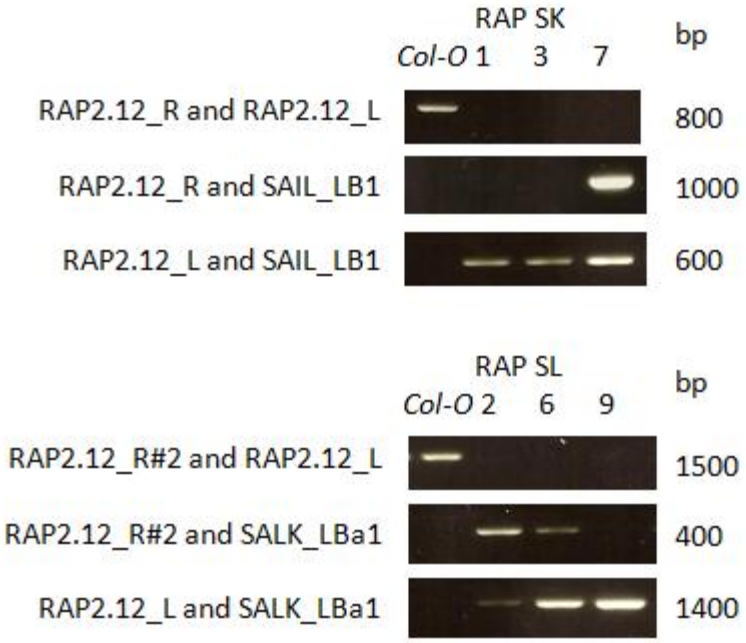

Figure 34 Genotyping the RAP insertion lines

Products from PCR amplified using primers from the left border (LB or LBa1) of the insertion and upstream and downstream of the insertion (RAP2.12 primers). RAP SK plants 1, 3 and 7 were homozygous for the insertion, and RAP SL plants 2, 6 and 9 were homozygous. Data are representative of two technical replicates.

5.4.3 RAP2.12 insertion lines show different levels of cell wall synthesis gene expression than wildtype

Reverse transcriptase polymerase chain reaction (RT-PCR) showed that *CELLULOSE SYNTHASE 3A* (*CES3A*), lignification *PEROXIDASE* (*PRX*), and *CINNAMATE-4-HYDROXYLASE* (*C4H*) have increased mRNA transcript levels in Arabidopsis lines with suppressed *RAP2.12* expression. *CINNAMOYL COENZYME A REDUCTASE* (*CCR*) had higher expression in *RAP2.12* insertion lines than in Col-0 (Figure 35).

The pixel intensities corresponding to the fluorescence intensity of the DNA bands were quantified using MatLab by converting the image of the gel to greyscale and using the *intensity value* function in MatLab. The pixel intensity in the image of the DNA bands was used to confirm differences in fluorescence intensity, which corresponds to the quantity of DNA in the band and therefore gene expression. Interestingly, *RAP2.12* down-regulation appeared to be more effective in the SAIL (RAP SL) insertion line; the pixel intensity was 101 compared to 80 in the SALK (RAP SK) line, suggesting the gene knock-out might be more effective. However this was not the case, as *C4H* gene expression, evaluated by RT-PCR, appeared enhanced in the SALK insertion line while the expression of *PRX* seemed to be up-regulated in the SAIL line. This suggested that the RAP2.12 might control gene expression in an on-off switch fashion and the slight differences observed in the pixel intensity do not reflect significant changes in gene expression.

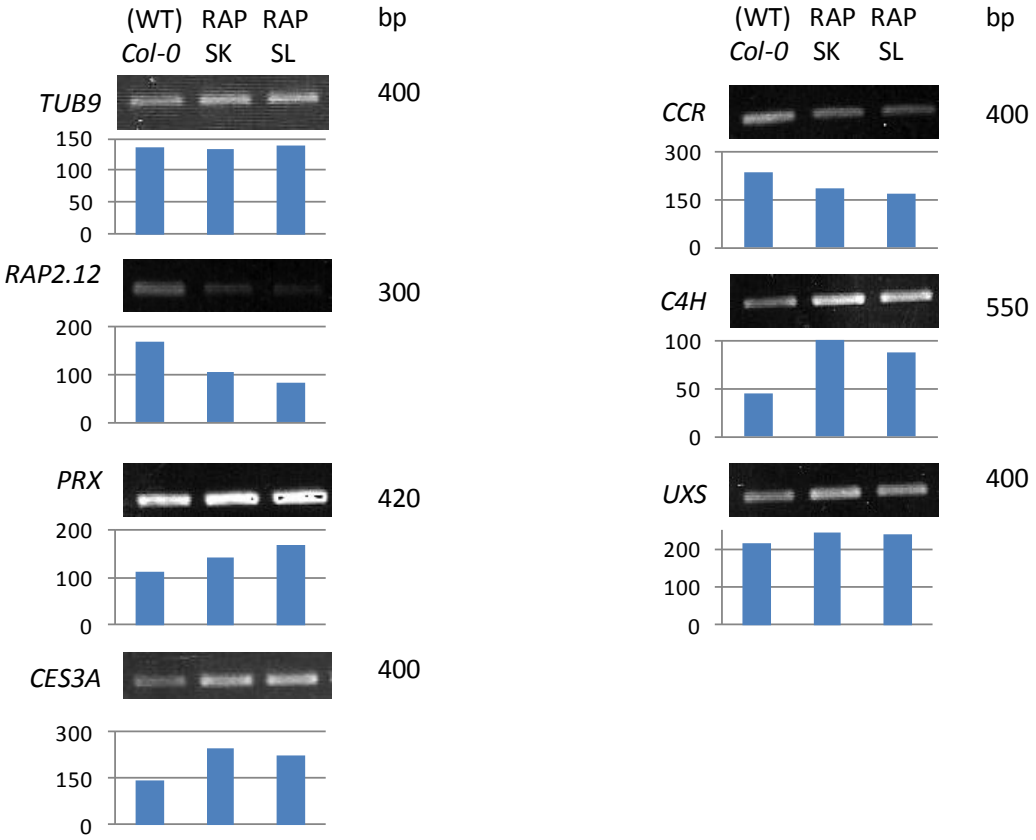

Figure 35 Semi-quantitative expression analysis of cell wall synthesis genes in *RAP2.12* Arabidopsis insertion lines. Pixel intensity of the bands on the gel was quantified in MatLab and is shown in graphs beneath each image. The images used were representative of two technical replicates; *RAP* insertion lines RAP SK and RAP SL are biological replicates.

5.5 mRNA transcripts involved in synthesis of all cell wall components have different expression in lines *ccr*, *c4h*, *prx* and *uxs*.

As a general trend, all the genes of lignin biosynthesis that could be identified in the EST were down-regulated in the lines where the phenylpropanoid pathway had been disrupted (Figure 36), with the exception of lignification-specific peroxidase *c4h* which was up-regulated. Our qRT-PCR results for all the genes found to be down-regulated in *ccr* are consistent with the AFLP analysis performed previously in *ccr* and *cad* tobacco plants, as well as in the *ccr/cad* double transformants (Leplé et al., 2007) so this seems to be a feature of lignin down-regulated lines in tobacco. The *ccr* line showed the highest changes especially with respect to *PAL*, *COMT* and *CCOMT*. The high changes observed in *prx* suggest the exsistance of a possible feedback mechanism in response to lack of polymerisation of monolignols in the peroxidase down-regulated line.

CES3A transcription was increased in all three lignin down-regulated lines (Figure 36). This suggests a compensatory mechanism whereby cellulose synthesis is increased in response to the limited lignin deposition. This was not reflected in the fractionation of cell wall components however. Similarly, only in line *ccr* was *SUSY*, the enzyme that feeds UDP-glucose into cellulose synthesis, up-regulated.

All the cell wall synthesis genes tested in this experiment with the exception of *CES3A* were down-regulated in *prx*. *prx* tobacco plants, though healthy, have fewer xylem vessels (Figure 19) and a smaller layer of vascular tissue in the stem (Figure 19). The reduction in secondary cell wall tissue is explained here by the down-regulation of both lignin and xylan synthesis genes seen in Figure 36.

The *uxs* line, containing less xylan that the wildtype, had elevated cellulose to xylan ratios and increased lignin compared to the wildtype K326 (Bindschedler et al., 2007). It was not possible to measure *Ces3A* expression in the K326 background as the primers were not compatible with K326 cDNA, indicating varietal variation in coding sequence. This also occurred for *CSLD* and *C3H*. Expression of the lignin related genes was around wild type levels or below, i.e. less than two-fold, which is not comparable with the high levels of lignin observed (Section 3.1; Bindschedler et al., 2007). As seen in Figure 36, the *uxs* line also showed general down-regulation of the genes for UDP-xylose provision including the non-target but redundant *UXS1*.

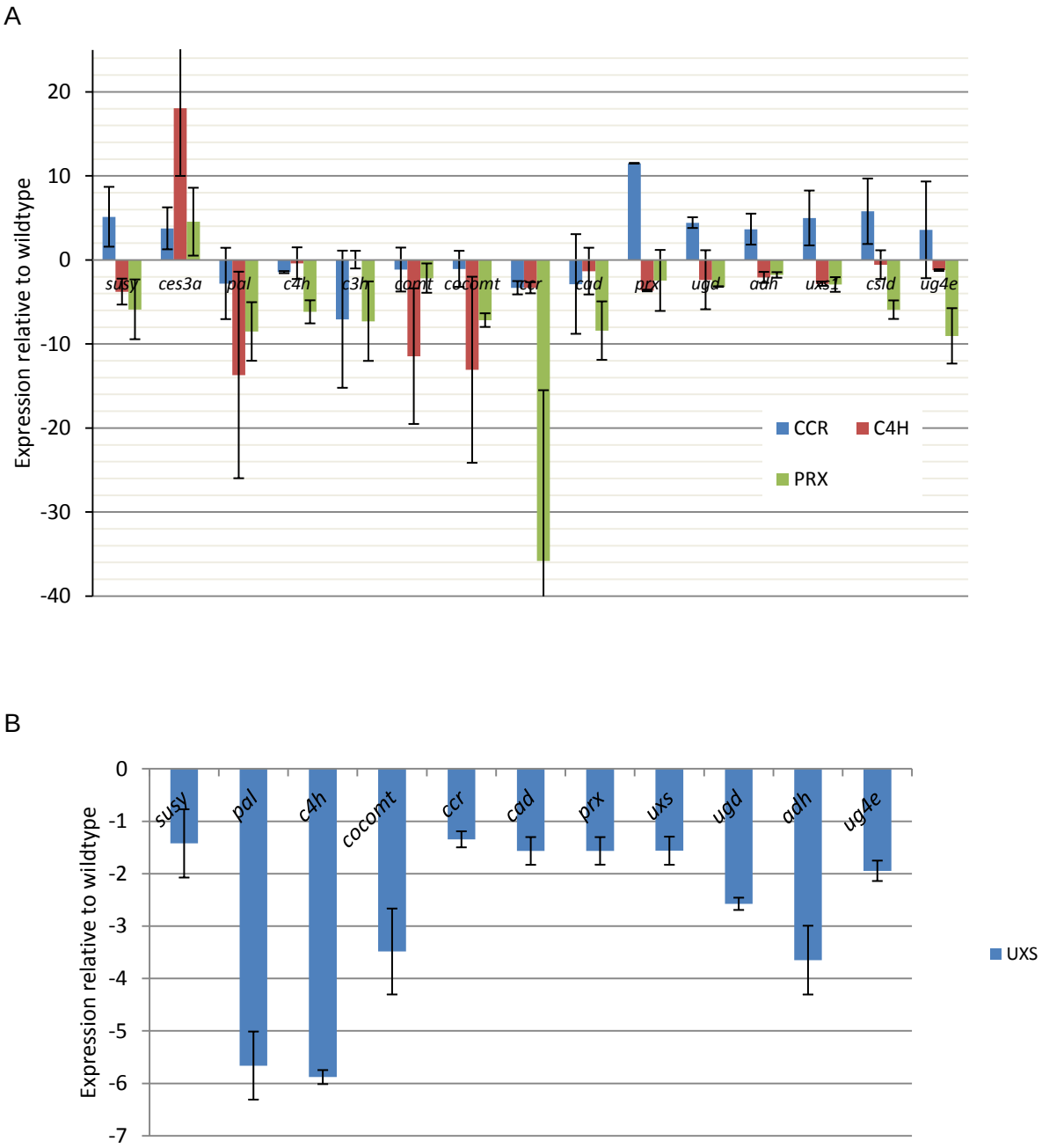

Figure 36 qRT-PCR expression analysis of cell wall synthesis genes in cell wall modified tobacco lines (Cook et al., 2011)
A Expression of cell wall synthesis genes in lines *ccr*, *c4h* and *prx*, relative to the wildtype NVS.
B Expression of cell wall synthesis genes in line *uxs*, relative to the wildtype K326.

Data is given in terms of change in abundance of the transcript for each gene as compared to the wildtype. Data are the average of 2 biological replicates, each containing 3 technical replicates. The bars indicate standard error. The reference gene was GAPDH.

SUCROSE SYNTHASE, SUSY; CELLULOSE SYNTHASE 3, CESA3; *PHENYLALANIE AMMONIA LYASE, PAL; CINNAMATE-4-HYDROXYLASE, C4H; 4-*

COUMARATE 3-HYDROXYLASE, C3H; CAFFEIC ACID O-METHYLTRANSFERASE, COMT; CAFFEOYL-CoA 3- O-METHYL TRANSFERASE, CCOMT; CINNAMOYL Co-A REDUCTASE, CCR.

5.6 Discussion

5.6.1 Xylogenic Tcyt cultures are a system for understanding xylogenesis and secondary cell wall synthesis

EST libraries have significantly contributed to understanding the complexity of the genes involved in cell wall production. In an analysis of 8962 ESTs from poplar suspension cells, presumably synthesizing primary cell wall, Lee et al. (2005) found that 62% of sequences could be fully annotated while 28% were of unknown function. The remaining 10% of EST sequences failed to show significant similarity to any proteins. These data are consistent with the EST database from the xylogenic tobacco cell culture analysed in this chapter (Cook et al., 2011).

Of the genes with known or predicted functions in the EST library, 13% were cell wall related genes (Table 11). This is high compared to wildtype BY-2 tobacco culture, in which cell wall related genes represent approximately 2% of the genes with known or predicted functions (Matsuoka et al., 2004). Combined with the proteomic analysis of the *Tcyt* culture described in Millar et al. (2009), the transcriptome analysis presented here has potential for novel gene discovery and annotation in cell wall biosynthesis and modification. As the EST library from the *Tcyt* tobacco line is enriched with secondary wall-related genes, and given that in the *Tcyt* culture 13% of ESTs were not significantly similar to any known DNA sequences and 23% represented unknown genes from unannotated genomes (Section 5.2), there is large scope to identify new genes involved in cell wall metabolism. All known lignin synthesis genes are present in the cDNA library which originated from the *Tcyt* culture, with the exception of *4-CL* and *PAL*. Low expression of these genes in xylem was confirmed by Genvestigator analysis, which shows that *PAL* and *4CL* have low expression in xylem relative to other cell wall synthesis genes, and higher expression in other tissues (Figure 33).

The value of the xylogenic *Tcyt* culture to deepen understanding of a complex family of cell wall-related genes was demonstrated by the cellulose synthases identified in the EST database. They were tomato and Arabidopsis *CELLULOSE SYNTHASE A3 (CESA3)* genes. Although *CESA3* was found to be expressed in xylem in other systems (Brown et al., 2005), it is thought to be involved in primary cell wall synthesis in both Arabidopsis and tomato (Burn et al., 2002). However the presence of *CESA3* homologues in the tobacco xylogenic culture suggests that, like aspen *PTCESA3*, it is likely to function as a secondary cell wall synthesis gene (Djerbi et al., 2005).

Similarly, representation of *GT14* and *CSLD* in the secondary cell wall synthesis library suggests a possible role for both of them in secondary cell wall polysaccharide biosynthesis. Their identification in the xylogenic culture described here is evidence in favour of their involvement in cell wall synthesis that builds on existing research, which is detailed below.

CSLD, a potential candidate for xylan synthase, was identified in the EST library and also found to be expressed by RT-PCR in wild type xylem tissue. Functional evidence for its role as a xylan synthase are controversial (Bernal et al., 2008; Samuga and Joshi, 2004). CSLD family members are co-localised to the cell membrane with glycosyl transferases known to be involved in cell wall synthesis (Bernal et al., 2008; Samuga and Joshi, 2004) and *CSLD* suppressed Arabidopsis plants have disrupted cell wall synthesis (Bernal et al., 2008). While down-regulation of wheat *CSLD* was shown to have no effect on the cell walls of wheat endosperm, which is rich in arabinoxylan (Nemeth et al., 2010), *CSLD* has also been identified in poplar secondary cell wall tissue (Samuga and Joshi, 2004). However, evidence from *Arabidopsis thaliana* indicates that members of the glycosyltransferase *GT43* family may be a xylan synthase (Peňa et al., 2007; Brown et al., 2007) and other research suggests there is no one xylan synthase, but rather a complex of enzymes (York and O'Neill, 2008).

Many glycosyltransferases have been implicated in hemicellulose biosynthesis; indeed any xylan synthase complex is likely to be made of several glycosyltransferases (York and O'Neill, 2008). Glycosyltransferases are enzymes which transfer monosaccharides from a nucleotide sugar to a glycosyl acceptor. Xylan synthesis as it is currently understood is described in section 1.2.2. Importantly, there are gaps in current understanding. Systems like the *Tcyt* xylogenic tobacco culture may unearth more xylan biosynthesis genes. One notable glycosyltransferase represented in the EST library is *GT14*, which has previously been identified in a xylogenic poplar system (Aspeborg et al., 2005). It has recently been suggested that the *GT14* family is likely to be involved in cell wall biosynthesis, based on bioinformatic analysis of *GT14* expression in both Arapidopsis and poplar, which showed that half the *GT14* family members are preferentially expressed in the stem or xylem (Ye et al., 2011).

5.6.2 RAP2.12 as a potential secondary cell wall regulator

RAP2.12 belongs to the AP2 family of ethylene responsive element binding transcription factors. Although the functions of all the RAP2 family members are uncertain, it is known that they have a similar expression to *AP2*. They are expressed

during inflorescence initiation and specification but also in the stem and leaves, indicating that they have an effect on vegetative growth too (Okamuro et al., 1997). They are involved in the ethylene response (Lin et al., 2008; Jofuku et al., 1994) and in drought stress tolerance (Lin et al., 2008).

Ethylene is known to be synthesised during tracheary element differentiation, probably in the apoplast of xylem elements, and is involved with regulation of the cambial stem cell pool size during secondary xylem formation (Pesquet, 2009). It is possible therefore that *RAP2.12* has a role in ethylene-regulated xylem differentiation. This research shows that it is xylem specific, and its expression correlates with secondary cell wall synthesis genes even to the extent that it is down-regulated in the xylem tissue of lignin suppressed lines *c4h, ccr* and *prx*. The involvement of *RAP2.12* in vascular tissue development, as a regulator of the transition from primary to secondary growth, has been suggested by Van Raemdonck et al. (2005), who identified the *RAP2.12* aspen homologue *ptaERF1* in the phloem tissue of developing aspen stems. However down-regulation of *RAP2.12* in Arabidopsis did not affect xylem structure.

There was a difference seen in expression of cell wall biosynthesis genes between wildtype Arabidopsis and *RAP2.12* insertion lines (Figure 35), which indicates its involvement in regulation of secondary cell wall synthesis at least at the transcriptional level. *RAP2.12* inhibits expression of *PRX, C4H, CES3A* and *UXS*, which are all up-regulated in the *RAP2.12* suppressed lines. Conversely *CCR* is down-regulated in the insertion lines. The regulation appears to be based on an on-off switch rather than a proportional scale, as the *RAP2.12* insertion lines have some *RAP2.12* expression and the response in cell wall biosynthesis genes is slight but appears significant.

5.6.3 Transcriptional interdependence between biosynthesis pathways of lignin, xylan and cellulose

Lignin synthesis genes in lines *ccr, c4h* and *prx* all showed reduced expression (Figure 36). This suggests that there might be feedback along the lignin biosynthesis pathway whereby expression of a gene at one stage of the pathway induces the expression of the others. This was also noted by Leple et al. (2007) in *CCR* and *CAD* down-regulated tobacco lines which show reduced expression of lignin biosynthesis genes.

Conversely the effects of lignin down-regulation on cell wall polysaccharide biosynthesis genes varied between lines *ccr, c4h* and *prx*. Cellulose and xylan

synthesis genes appeared over-expressed in line *ccr* while in *prx* and *c4h* xylan synthesis genes were down-regulated. This suggests the existence of a compensatory feedback mechanism between cell wall polymers, in which *CCR* is a significant player. *CCR* reduces *p*-coumaroyl CoA, feruloyl CoA and sinapoyl CoA into their aldehydes, mid-phenylpropanoid pathway. *CCR* down-regulation has the opposite effect on cellulose and xylan synthesis to *C4H* and *PRX* which occur at the start of the phenylpropanoid pathway and the end of lignin synthesis respectively. To be noted is the effect of *CCR* knockouts or antisense manipulation that resulted in increased hemicellulose and cellulose in Arabidopsis (Ruel et al., 2009) while the opposite occurred in poplar (Leple et al., 2007), so the effects of *CCR* down-regulation appear to vary between species.

Cell wall synthesis regulation is a research topic of great interest and is not fully understood. Current understanding revolves around AC elements in the promoter sequences of lignin synthesis genes, and little is known of the effect of cell wall transcription factors on individual cell wall synthesis genes. Specificity has been shown to occur with respect to lignin synthesis regulation however. Pine MYB transcription factor PtMYB4 was over-expressed in Arabidopsis, causing fourfold increase in *CCOMT* expression while *4-CL1* expression remained at the wildtype level (Zhong and Ye, 2009).

Clearly *CCR* down-regulation triggered a response in cellulose and hemicellulose biosynthesis that ded not occur by down-regulating *C4H* and *PRX*. It is possible to infer from this that differential feedback mechanisms exist from the lignin biosynthesis pathway to the cellulose and xylan synthesis pathways. As lines *ccr, c4h* and *prx* all had reduced lignin contents (Table 6), any feedback that occurs is not necessarily a result of final lignin content. Further study of known cell wall transcription factors such as MYBs and NACs in *ccr* compared to *c4h* and *prx* would be the first step to valuable understanding of the details of cell wall synthesis regulation.

Hemicellulose down-regulation caused by suppression of *UDP-GLUCURONATE DECARBOXYLASE* resulted in reduced expression of all cell wall biosynthesis genes analysed. However the difference in expression between wildtype and *uxs* transcript levels in *SUSY, CCR, CAD, PRX* and *UXS* is less than twofold and is not significant. *PAL, C4H, CCOMT, UGD, ADH* are all significantly down-regulated in *uxs.* Down-regulation of lignin synthesis genes did not reduce the final lignin content of *uxs* stem AIM (Table 6). Possible reasons for this will be explored in chapter 6.

6 Discussion

6.1 Feedback signalling exists within the lignin biosynthesis pathway

There have been few studies on the effect that lignin down-regulation has on the cell wall, either at the transcriptional level or on the deposition of cell wall polymers. In the data presented in this study, there are clear indications of cross talk between the lignin and xylan pathways. When *CCR, C4H* and *PRX* are down-regulated, genes encoding other members of the phenylpropanoid pathway have reduced transcription. The lignin content in the cell wall of these lines is reduced (Table 6; O'Connell et al., 2002; Blee et al., 2003; Blee et al., 2001) which is consistent with the suppression of lignin biosynthesis genes. This data is also consistent with other studies of lignin down-regulated plants. In Arabidopsis lines in which *C3H* was down-regulated by cosuppression, Klason lignin content was reduced from 18.5% in the wildtype to 11.7% in the insertion line and phenylpropanoid pathway genes were suppressed. (Abdulrazzak et al., 2006). In tobacco, *4-CL* down-regulation caused the lignin content to drop from 23.4% to 20.3% (Kajita et al. 1996) but a much bigger reduction of lignin content of 40% was achieved by antisense *4-CL* suppression in aspen (Li et al., 2003). Antisense down-regulation of *CCR* and *CAD* in alfalfa generated plants with as little as 12% and 15% acetyl bromide lignin content respectively, compared to 17.5% in the wildtype plant (Jackson et al., 2008). With the exception of Abdulrazzak et al. (2006), none of the other authors analysed gene expression as well as cell wall composition. An Arabidopsis *CCR1* knockout line also showed reduced lignin content, though the reduction was smaller; from 15.1% in the wildtype to 13.8% in the GC-MS (Ruel et al., 2009).

Intra-pathway down-regulation in response to disruption of a step in lignin biosynthesis is also consistent with previous research. Similar down-regulation of phenylpropanoid pathway genes was observed in *CCR* and *CAD* down-regulated tobacco (Leplé et al., 2007), and as described above lignin biosynthesis genes were down-regulated in *C3H* suppressed Arabidopsis (Abdulrazzak et al., 2006). Together with the results presented here, which show low expression of lignin synthesis genes in all three lignin down-regulated lines, current research suggests that the lignin biosynthesis pathway is regulated by a negative feedback mechanism. No whole pathway feedback mechanism has been identified, however evidence of a specific negative feedback loop within the phenylpropanoid pathway was found by Blount et al. (2002), who showed that *PAL*

activity was reduced in tobacco plants with down-regulated *C4H* but the same was not true in reverse. Where C4H did not synthesise p-coumaric acid, PAL did not synthesise the C4H substrate cinnamic acid; but when PAL did not produce cinnamic acid there was no down-regulation of C4H activity.

Suppression of all the genes in a pathway reduces the possibility of an excess of the substrate of the silenced enzyme, or the build-up of products of alternative pathways such as the biosynthesis pathways of flavonoids, tannins and anthocyanins, in which phenylpropaoid pathway enzymes occur. High levels of free phenolics, in particular caffeic acid esters, another phenylpropanoid pathway product, were observed in tobacco lines with antisense suppression of *CAD* and *CCR* (Chabannes et al., 2001). One of the *CCR* down-regulated lines was dwarfed, and had abnormal, necrotic leaves. They suggested increased levels of monolignol derivative and hormone mimic β-5-dehydrodiconiferyl alcohol caused this abnormal growth. Chabannes et al. (2001) did not analyse transcript levels of phenylpropanoid pathway genes. A rewarding avenue of the work presented in this report on lignin down-regulated lines *prx*, *ccr* and *c4h* would be metabolite profiling to observe if the cross-pathway suppression of lignin biosynthesis genes prevents the build up of free phenolics observed in the Chabannes *ccr* and *cad* lines.

6.2 Feedback signalling exists between lignin and cell wall polysaccharide biosynthesis pathways

The qRT-PCR analysis data presented here presents opposite effects on the transcriptional response to lignin down-regulation by suppressing expression of genes at different stages of the pathway. Results show down-regulation of xylan synthesis genes in *prx* and *c4h* but up-regulation of both cellulose and xylan synthesis genes in *ccr* (Figure 36). Increasing cellulose and xylan biosynthesis genes may be the first step in a compensation mechanism to replace lignin in *ccr.* This was the case in Arabidopsis *CCR* and *CAD* down-regulated lines, in which transcriptome analysis showed an increase in primary cell wall gene transcripts for expansin, extensin and polygalacturonase (Leple et al., 2007). Also this study, the only published study analysing both gene expression and cell wall composition in lignin modified plants, showed that despite down-regulation of gene expression of cell wall carbohydrate metabolism genes *UGD, PME* and a β-glucosidase, there were increased levels of

non-cellulosic monosaccharides arabinose and xylose. This is indicative of multiple levels of regulation for cell wall synthesis.

As seen in Figure 36 and 7, genes encoding hemicellulose and cellulose synthesis proteins are suppressed in the *c4h* and *prx* lines, with a fold change of between two and six in *c4h,* and two and ten in *prx*. Conversely in line *ccr*, polysaccharide biosynthesis genes are up-regulated between four- and six-fold. The exception is *CELLULOSE SYNTHASE (CESA3)*, which is up-regulated in all three lines. The up-regulation of *CES3A* in all three lignin down-regulated lines may be the transcriptional level compensatory response to the reduction in lignin. Cellulose is the major secondary cell wall polysaccharide, making up 40% of the cell wall while hemicellulose makes up 20%. *CESA3* is the cellulose synthase, and its up-regulation is more likely to increase cellulose content than if *SUCROSE SYNTHASE (SUSY)* was up-regulated because there are other pathways which supply CESA3 with UDP-glucose. However the down-regulation of *SUSY* in lines *prx* and *c4h* is significant; fourfold in *c4h* and sixfold in *prx.* If the increased level of *CESA3* transcription translates into more proteins, its substrate UDP-glucose may not be greatly increased so the up-regulation of *CESA3* will not translate into more cellulose.

There is no compensation feedback mechanism where polysaccharides replace lignin in *ccr* and *c4h*. This is similar to the results of AFLP analysis of cDNA from the xylem of tobacco lines with *CCR* or *CCR* and *CAD* down-regulation, where non-cellulosic cell wall polysaccharide genes *UDP-GLUCOSE DEHYDROGENASE* and *PECTIN METHYLESTERASE* were down-regulated (Leple et al., 2007). The cell wall synthesis response to lignin modification must depend on the individual gene and corresponding enzyme targets rather than the reduction in lignin content, as the lignin contents of *ccr, c4h* and *prx* are not significantly different from one another.

6.3 RAP2.12 is a potential cell wall synthesis regulator

RAP2.12 belongs to the AP2 family of ethylene responsive element binding transcription factors. Although the functions of all the RAP2 family members are uncertain, it is known that they have a similar expression to *AP2*. They are expressed during inflorescence initiation and specification but also in the stem and leaves, indicating that they have an effect on vegetative growth too (Okamuro et al., 1997). They are involved in the ethylene response (Lin et al., 2008; Jofuku et al., 1994) and in drought stress tolerance (Lin et al., 2008).

Ethylene is known to be synthesised during tracheary element differentiation, probably in the apoplast of xylem elements, and is involved with regulation of the cambial stem cell pool size during secondary xylem formation (Pesquet, 2009). It is possible therefore that *RAP2.12* has a role in ethylene-regulated xylem differentiation. This research shows that it is expressed highly in the xylem, and has expression highly correlated with secondary cell wall synthesis genes even to the extent that it is down-regulated in the xylem tissue of lignin suppressed lines *c4h, ccr* and *prx*. The involvement of *RAP2.12* in vascular tissue development, as a regulator of the transition from primary to secondary growth, has been suggested by Raemodonck et al. (2005), who identified the *RAP2.12* aspen homologue *ptaERF1* in the phloem tissue of developing aspen stems. However down-regulation of *RAP2.12* in Arabidopsis did not affect xylem structure.

There was a difference seen in expression of cell wall biosynthesis genes between wildtype Col-0 and the *RAP2.12* insertion lines (Figure 35) which indicates the involvement of *RAP2.12* in regulating secondary cell wall synthesis. A possible model for the action of *RAP2.12* on cell wall synthesis at the transcriptional level is given in **Error! Reference source not found.**. *RAP2.12* inhibits expression of *PRX, C4H, ES3A* and *UXS*, which are all up-regulated in the *RAP2.12* suppressed lines. Conversely *CCR* is down-regulated in the insertion lines.

6.4 Post transcriptional regulation of cell wall biosynthesis genes: possible involvement of the splicing factor *RSZ33*

Cell wall fractionation of the transgenic tobacco lines (shown in Section 3.1) reflects neither polysaccharide down-regulation, as expected by qRT-PCR results in *c4h* and *prx* which show twofold or more down-regulation in xylan synthesis genes, nor up-regulation in *ccr,* in which xylan synthesis genes were up-regulated (Figure 36). Similarly, xylan down-regulation by antisense insertion in *UDP-GLUCURONATE DECARBOXYLASE* leads to conflicting results. In transgenic down-regulated line *uxs,* transcript levels indicate lower levels of lignin and cellulose genes but lignin content is increased and cellulose content does not change. This could be because of redundancy of enzymes within the cell wall synthesis pathway, many of which are part of large, closely linked gene families. For example, among the *UXS* down-regulated tobacco lines generated alongside *uxs,* the line presented in this study, had low xylose content but a small decrease in *UXS* activity compared to many of the other transgenic

plants (Bindschedler et al., 2007). This suggests that that the levels of cellulose, xylan and lignin are not wholly dependent on transcription. There must be post-transcriptional regulation of gene expression, as transcription does not determine final cell wall composition.

Splicing factors are essential for processing pre-mRNA in preparation for translation. Non-coding regions of pre-mRNA are spliced out, and some genes have alternative splicing sites which allow two isoforms to be synthesised from the same gene. This whole process is part of post-transcriptional regulation of gene expression; splicing factors can determine what, if anything, is synthesised from an mRNA (Buchanan et al., 2000b). One of the genes identified in the list of ESTs expressed in the *Tcyt* culture and proven to have xylem-specific expression in Genevestigator (Hruz et al., 2010), is the splicing factor RSZ33.

Lopato et al. (2002) proposed that RSZ33 has a role in spliceosome assembly and Kalyna et al. (2003, 2006) confirmed its involvement in splicing by observing abnormal splicing patterns in the pre-mRNA of *RSZ33* itself and in three other genes in Arabidopsis lines over-expressing *RSZ33*. The plants also had abnormal polarization of cell division, greater cell expansion and up-regulation of *AIR3*, a gene involved in auxin-induced lateral root formation and proposed to be involved in changing cell wall structure (Kalyna et al., 2003).

Cell expansion in *RSZ33* over-expressers is abnormal (Kalyna et al., 2003), suggesting abnormalities in cell wall deposition. *RSZ33* has high expression in xylem and in a cell culture with constitutive secondary cell wall synthesis. It is up-regulated in *uxs*, in which lignin synthesis genes are down-regulated but lignin deposition is increased, but down-regulated in lines *ccr, c4h* and *prx* in which both lignin synthesis genes and lignin deposition is decreased. It is possible to speculate that splicing factor *RSZ33* is one of the splicing factors that prepare secondary cell wall synthesis pre-mRNA for translation, and when lignin synthesis is interrupted, it is down-regulated due to the same negative feedback suggested above but when lignin synthesis is increased in compensation for reduced xylan, its transcription is increased so more lignin biosynthesis mRNA is processed.

6.5 Interaction between vascular tissue formation and cell wall synthesis

Both *prx* and *uxs* lines show a reduction in vessels (Figure 19). *REVOLUTA*, a transcription factor known to influence vascular development (Emery et al., 2003) was up-regulated in *prx* but not *uxs* (Figure 32). Two other vascular active transcription factors, *SVP* and *RSZ33*, were up-regulated in *uxs*. *RAP2.12*, which has a role in ethylene signaling active in the terminal stages of xylogenesis, was down-regulated in all lines.

It seems that secondary cell wall synthesis in the xylem is related to xylem development, deposition or patterning. However it is not clear if the changes in cell wall caused altered xylem vessel element shape, which in turn triggered differences in vascular pattering transcription factors; or if there was a direct feedback mechanism from the down-regulated cell wall biosynthesis genes to vascular patterning genes, which caused modified xylem structure. Ethylene is involved in both xylogenesis and stress induced cell wall synthesis regulation. Arabidopsis *CESA3* mutant *cev1* has low cellulose content and constitutively high ethylene and jasmonate production. When ethylene and jasmonate signaling is interrupted, the low cellulose content phenotype is recovered (Ellis et al., 2002). That study suggested ethylene and jasmonate have an inhibitory effect on cell wall synthesis. Ethylene also affects wood formation and increasing ethylene production during wood formation changes cell wall composition. In the absence of ethylene, indoleacetic acid (IAA) promotes non-cellulosic polysaccharide deposition while increasing ethylene production caused IAA to induce cellulose deposition (Eklund, 1991).

The *RAP2.12* Arabidopsis insertion lines have normal vascular structure (data not shown) and slight differences in *CES3A*, *PRX* and *C4H* expression, seen in Figure 35. This data seems to suggest that changing *RAP2.12* expression alone does not have the effect on the plant that the large changes in lignin content has, and cell wall modification caused differences in vascular phenotype. Additionally irregular xylem Arabidopsis plants have collapsed xylem vessels in response to down-regulated cell wall synthesis genes (Taylor et al., 2003; Brown et al., 2005; Brown et al., 2007, Peňa et al., 2007). However, RAP2.12 is not known to be an important regulator of xylogenesis and vessel elements collapse if the cell wall is not strong enough to withstand osmotic pressure. Expression analysis of cell wall genes in plants with reduced or knocked-out vascular development transcription factors such as VASCULAR-RELATED NAC-DOMAIN 6, which can induce transdifferentiation of cells

into metaxylem-like vessel elements (Kubo et al., 2005) must be carried out to clarify the link between cell wall synthesis and vascular phenotype.

On the other hand there is evidence that *RAP2.12* regulates, directly or indirectly, the expression of cell wall biosynthesis genes such as *PRX, CCR, C4H, UXS* and *CESA3* in Arabidopsis (Section 5.4.2). *RAP2.12* appears to have a role in cell wall synthesis; but in this study there is no evidence for its involvement in xylogenesis.

6.6 Lignin and xylan down-regulation modification does not increase cellulose deposition but does affect cell wall composition

As glucose is the most industrially valuble monosaccharide in the cell wall, it is of particular interest if cellulose deposition, and consequentially glucose content, increases in response to disruption of the biosynthesis pathways of other cell wall components.

The results presented in Table 6 suggest that there is no compensation mechanism where lignin biosynthesis is down-regulated to increase the levels of cellulose. While lignin content is reduced in *ccr, c4h* and *prx*, there is no difference in the polysaccharide content in these lines. These results confirm the evidence from a current review of the literature; which is discussed in Section 3.8.2.

Conversely the *uxs* line, in which *UDP-GLUCURONATE DECARBOXYLASE* down-regulation causes xylan deposition to halve, shows lignin content increasing from 21% in the K326 wildtype to 30% in *uxs*. The compensatory increase in lignin content in plants with reduced polysaccharide content has been observed previously: in poplar trees with reduced xyloglucan content due to expression of an *Aspergillus* xyloglucanase, there was an increase in cellulose content but a decrease in lignin content (Park et al., 2007); and hemicellulose down-regulated poplar lines had increased lignin content and reduced cellulose (Lee et al., 2009).

Arabidopsis lines with low endo-1,4-β-glucanases had very low pectin content. Endo-1,4-β-glucanases are not pectin synthesis genes; they hydrolyse β-1,4-linkages in hemicellulose and cellulose, acting on the β-1,4-linkages behind un-substituted glucose residues (His et al., 2001). This is evidence of hemicellulose and cellulose modification

having an effect on pectin content, a reactive measure to increase pectic cross-linking to replace some of the structural integrity lost by the reduction in cellulose content.

In the lignification specific peroxidase down-regulated line (*prx*) there was an increase in *S* over *G* units revealed by immunocytochemistry (Kavousi et al., 2010). This is due to the manipulation of the polymerisation step. This may indicate differential specificity of more than one peroxidase operating in vascular tissue; the peroxidase down-regulated in this line is *TOBACCO PEROXIDASE 60* (*TP60*) which appears to have more activity towards guaiacyl units. An increase in *S* over *G* units has been found for tobacco lines down-regulated for *PAL.* Similarly, down regulation of the downstream enzymes of monolignol biosynthesis, CONIFERYLALDEHYDE 5-HYDROXYLASE (F5H), CCR and CAD (Chabannes et al., 2001a,b; O'Connell et al., 2002) leads to limited effects on total lignin but with drastic and opposing changes in S/G ratios (Anterola and Lewis, 2002).

6.7 Pretreatment of biomass with *Phanerochaete chrysosporium* as a most reliable method of improving glucose extractability

Lignin down-regulation by preventing polymerization of monolignols has a big effect on saccharification. But down-regulating other cell wall synthesis genes does not improve cellulose accessibility, despite the lignin content being similar. As this is due neither to differences in cell wall synthesis gene expression nor cell wall composition (Table 6, Figure 36 and 37), it is likely to be due to differences in hemicellulose and pectin monosaccharide composition which change to improve cross-linking within the wall. This was discussed in Chapter 3. However, simply down-regulating lignin content will not make biomass more amenable to cellulose hydrolysis. Reducing lignin content by down-regulating *TP60*, a peroxidase involved in final polymerization of monolignols, increases sugar release by 30%, but suppression of *C4H* and *CCR* did not have the same effect (Figure 22).

Fungal pretreatment is a reliable method of improving cellulose accessibility in biofuel feedstocks. The results are consistent: in every material used as a substrate, saccharification was increased after pretreatment (Figure 28 to 31). Additionally, while use of genetically engineered plants is not met with public approval, fungal pretreatment results in no perceived ethical dilemmas.

The difference between line *prx* after pretreatment and the wildtype NVS with no pretreatment is enormous when considered on a large scale (Table 13). Before pretreatment, it would require 252.96 g of NVS cell wall material to extract 100g glucose. After pretreatment, 145.52 g would be necessary. However only 103.93 g of pretreated *prx* cell wall material is required for 100g glucose. Similarly, wildtype K326 requires only 116 g cell wall material to yield 100g glucose after fungal pretreatment, but with no pretreatment 302 g are necessary.

Table 13 Quantity of acetone insoluble cell wall material (AIM) necessary to produce 100g of glucose by saccharification

Line	Grams of AIM required for 100g glucose	
	Pretreated with *P. chrysosporium*	No pretreatment
WT (NVS)	146	253
PRX	104	156
CCR	222	628
C4H	159	369.84
WT (K326)	117	303
UXS	128	234

6.8 Future work

6.8.1 Reconciliation of 'insoluble residue' and acetyl bromide lignin

Two methods of lignin quantification were shown in Table 6. The over-estimation of lignin content by the Klason method is a known problem, and is due to proteins and other alkali and acid insoluble residues co-precipitating with lignin after acid hydrolysis (Anterola and Lewis, 2002). Klason quantification, which is labelled as insoluble residue, resulted in higher estimations of lignin content than acetyl bromide lignin analysis in the cell wall modified lines. This shows that though there are no significant differences in polysaccharide content between NVS, *ccr*, *c4h* and *prx*, there are different levels of non-lignin alkali- and acid- insoluble residue present in the cell wall.

The non-lignin insoluble residue which is not accounted for by the acetyl-bromide lignin assay is not made up of free non-lignin phenolics as these would be detected by UV absorbance. Structural proteins such as expansins and extensins are covalently bonded to acidic residues, such as uronic acid, via dimerization of tyrosine residues to form isodityrosine (Fry and Miller, 1989). It is likely that the insoluble residue comprises these structural proteins in an attempt to compensate for the lack of structural integrity lignin provides. Metabolic analysis must be carried out on this residue in order to ascertain what the insoluble residue comprises, and what is present in *prx*, *ccr*, *c4h* and *uxs* that is not present in wildtype.

6.8.2 Synthesis of further improved biofuel feedstock by combining down-regulation of *prx* and *uxs*

UDP-GLUCURONATE DECARBOXYLASE down-regulation, causing reduced xylan content, also improves saccharification. Given the normal phenotype of *prx* and *uxs*, generating the double mutant may result in a healthy plant which is still more amenable to cellulose hydrolysis. The *uxs* line has higher lignin content than the wildtype, while *prx* shows marked lignin down-regulation. Suppressing lignin and xylan synthesis in the same plant has not been attempted before.

Though *uxs* and *prx* appear to be good candidates for a biofuel feedstock due to the improvement in saccharification properties, tobacco may not be the ideal crop to chose. The next step in utilization of this information is to transfer the transgenesis into a more appropriate crop, such as wheat or a short rotation coppice crop such as poplar. Wheat stem is often burned after harvesting, but it could be used for second generation biofuel production if the cellulose was more easily accessible. Current data suggests transferring the transgenesis to another plant, even a monocot such as wheat, may yield positive results as pectin down-regulated wheat with improved saccharification efficiency was generated after the concept was proved in Arabidopsis plants (Lionetti et al., 2010).

6.8.3 Optimisation of *Phanerochaete chrysosporium* pretreatment

There were some limitations to the research presented in the chapter. The incubation time was not optimised due to time restraints. The protocol adapted for this work, designed by Tien and Kirk (1988), states that lignin peroxidase activity is at its maximum on day 6. However other research has used pretreatment times ranging from 7 days (Zeng et al., 2010) to 12 weeks (Blanchette et al., 1992). Incubation time varied according to the objectives of the pretreatment, the protocol used and the substrate.

Given that cell wall modifications further complicate the issue, continuation of this work would require investigation into the effects of incubation time on the lignin depolymerisation and saccharification properties of each plant line.

6.8.4 Confirmation of *RAP2.12* as a cell wall regulator

It seems that *RAP2.12* has an effect on the expression of cell wall synthesis genes. A model of suggested interactions, at the transcriptional level, is proposed in Figure 37. It includes the up-regulation of *CCR* by *RAP2.12* and the down-regulation of *UXS, CESA3, PRX* and *C4H* by *RAP2.12.* This is based on the fact that when *RAP2.12* is absent, transcription of *CCR* is suppressed but *UXS, CESA3, PRX* and *C4H* have increased expression. Additionally the model includes the possible results in terms of lignin, xylan and cellulose synthesis in response *PRX, C4H* and *CCR* down-regulation. Lignin synthesis is stimulated by the expression of genes in its pathway. Xylan synthesis genes are down-regulated when *PRX* or *C4H* expression is disrupted, but up-regulated when *CCR* is suppressed. This model needs to be confirmed by quantitative RT-PCR and at the metabolic level by analysis of lignin and hemicellulose content in *RAP2.12* suppressed lines.

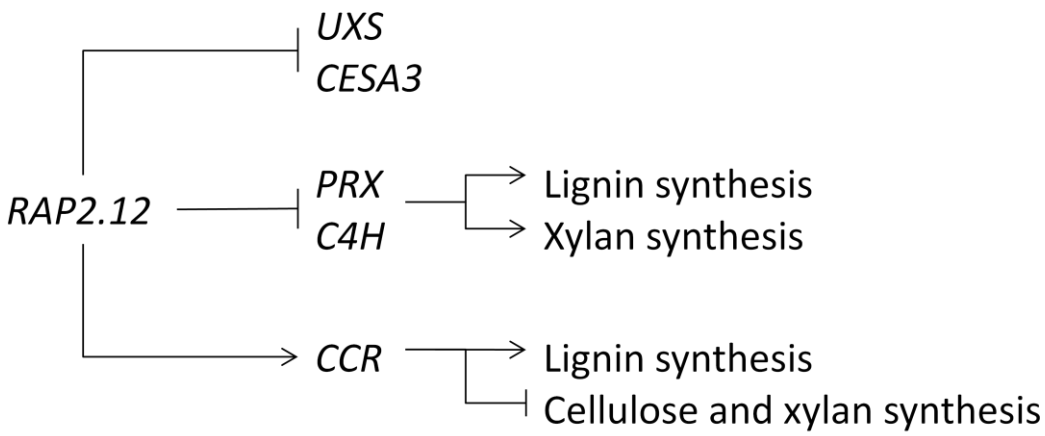

Figure 37 Model for the transcriptional response to down-regulation of *PRX, C4H* and *CCR* in tobacco stem cell wall material.

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